XXXIX. SCYLLITOL IN SELACHIAN ONTOGENY.

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One of the principal aims of chemical embryology must certainly be to find out, in the case of any given egg, exactly what the embryo is provided with by the maternal organism. Anything with which it is not provided at the start must either be absorbed from the environment or synthesised during development, and it is important to know how much of this kind of work the embryo has to perform during its morphogenesis. We know, for example, that the eggs of marine invertebrates are not supplied with enough water or enough ash to make the complete embryo, while those of fishes have enough ash but not enough water. With regard to organic constituents also the same distinction can be made. As far as we know all eggs contain sufficient protein, and in no case does protein have to be manufactured from other nitrogenous bodies. Probably the same holds true of carbohydrate, but undoubtedly certain fishes and gastropods have to synthesise fatty acids during their ontogeny, and there is evidence that in the eggs of birds, for instance, some 15 % of the total cholesterol which the embryo requires has to be formed from other substances. Lipins, on the other hand, judging from the behaviour of ether-soluble phosphorus, are always present in ample amount at fertilisation, though the range of species for which we have information is not large. The two most outstanding instances of substances which are not provided by the maternal organism are pigments—thus no egg contains haemoglobin—and cycloses.

In a previous paper [Needham, 1924] it was shown that the *i*-inositol which is found in all the tissues of the newly hatched chick has to be synthesised by the embryo during the three weeks of its development. This observation had been previously made by Klein [1909] but the course of formation of the cyclose by the chick was followed by an estimation-method, which, though it left much to be desired, was an improvement on any of the techniques which had been at the disposal of the earlier workers.

The inositol of birds and mammals is not the only one which occurs in animal tissues. Of its seven possible stereoisomeric modifications, one other is known, scyllitol, first isolated from elasmobranch fishes by Stadeler and Frerichs [1858]. This body is identical with the quercinitol which has been obtained from acorns, and with the cocositol of the palm-tree [Müller, 1912]. In view of the fact that the cycloses have recently acquired considerable

physiological importance through the identification of *i*-inositol with (Wildier's) Bios I by Eastcott [1928], and for the general embryological reasons already mentioned, it was thought worth while to investigate the origin of scyllitol during the development of a selachian fish. The material, collected at the Millport Marine Biological Station, consisted of eggs and embryos of *Acanthias vulgaris* (the "spur-dog") and *Scyllium canicula* (the "rough-dog").

The method of isolation of the scyllitol embodied the most useful parts of various previous methods. The eggs or minced embryos were preserved in acetone and, when the time came, were extracted repeatedly with acetone. The combined extracts were then freed from acetone by a vacuum distillation and the aqueous solution extracted several times with ether in order to remove pigments and substances of a fatty nature. Most of the pigment of the eggs and some of the pigment from the embryos was thus removed. The solution was freed from ether by evaporation and protein precipitated by boiling with acetic acid at a p_H at which bromocresol purple was just green. A saturated solution of neutral lead acetate was then added after neutralisation and the precipitate, which was usually small in amount, filtered off and discarded. To 100 parts of the filtrate were now added 60 parts of a saturated solution of basic lead acetate, 20 parts of a 40 % solution of cadmium nitrate, and 5 parts of concentrated ammonia, for the cycloses are completely carried down on the precipitate of lead hydroxide and the double salt of cadmium and lead which is formed. The precipitate was then filtered off, washed, suspended in distilled water, and decomposed with hydrogen sulphide. The lead sulphide was next removed by filtration, three or four extractions with water at 70° being required to wash the cyclose completely away from it. A second treatment with hydrogen sulphide was sometimes necessary, but, if not, the solution was evaporated down to about 10 cc. and poured into 20 times its volume of absolute alcohol, when the scyllitol separated in the form of a microcrystalline powder. After being allowed to stand overnight it was filtered off and weighed, a correction being made for whatever ash the product contained, as the cycloses, being insoluble in all organic solvents, are rather difficult to free from salts.

In some cases the simpler method of Rosenheim [1917] was used, in which the original extracts, freed from acetone, and allowed to stand at a low temperature, deposit crystals of scyllitol. A sample of scyllitol prepared in the course of this work melted at 330°. The following results were obtained.

Acanthias vulgaris:

Exp. B_I. 8 *yolks of undeveloped eggs* (the perivitelline liquid, which was included, is very small in amount):

Total weight, 167 g.; *i.e.* 20·8 g. each egg wet weight.
Total dry weight, 53 g.; *i.e.* 6·63 g. each egg dry weight.
Percentage of water, 68·2.
Scyllitol isolated, 2·0 mg.;
i.e. 0·25 mg. per egg;
1·2 mg. per 100 g. wet weight;
4·17 mg. per 100 g. dry weight.

Exp. D. 3 *yolks of undeveloped eggs:*

Total wet weight, 75·7 g.; *i.e.* 25·2 g. each wet weight.
Total dry weight, 33 g.; *i.e.* 11 g. each dry weight.
Percentage of water, 56·5
Scyllitol isolated, traces only, insufficient for weighing.

Exp. A_m. 2 *yolks of* 23 *cm. embryos:*

Total wet weight, 37 g.; *i.e.* 18·5 g. each wet weight.
Total dry weight, 12·6 g.; *i.e.* 6·3 g. each dry weight.
Percentage of water, 66.
Scyllitol isolated, 12·0 mg.;
i.e. 6·0 mg. per egg;
32·4 mg. per 100 g. wet weight;
95·1 mg. per 100 g. dry weight.

Exp. E. 2 *embryos of* 23 *cm. length* (corresponding to the twentieth month of development out of twenty-one [Ford, 1921]):

Total wet weight, 80 g.; *i.e.* 40·0 g. each wet weight.
Total dry weight, 12·5 g.; *i.e.* 6·25 g. each dry weight.
Percentage of water, 84·1.
Scyllitol isolated, 52·5 mg.;
i.e. 26·25 mg. per embryo;
65·7 mg. per 100 g. wet weight;
420·0 mg. per 100 g. dry weight.

Exp. F. 12 *embryos of* 23 *cm. length:*

Total wet weight, 496 g.; *i.e.* 41·3 g. each wet weight.
Total dry weight, 61 g.; *i.e.* 5·09 g. each dry weight.
Percentage of water, 87·6.
Scyllitol isolated, 292·2 mg.;
i.e. 24·3 mg. per embryo;
59·0 mg. per 100 g. wet weight;
480·0 mg. per 100 g. dry weight.

Exp. G. *Adult muscle:*

From 47·75 kg. wet weight (9·55 kg. dry weight) 5·75 g. of scyllitol were obtained; this yield was almost exactly the same as that obtained from 50 kg. by Rosenheim [1917] and Müller [1912]: 0·012 mg. per 100 g. wet weight; 0·06 mg. per 100 g. dry weight.

Scyllium canicula:

Exp. C. 4 *egg-contents* (*yolk and jelly*):
(As *Acanthias vulgaris* is an ovoviviparous form, retaining its eggs and embryos within the uterus until the end of their development, it might be argued that the supply of scyllitol was derived from the mother. *Scyllium*, however, is oviparous.) Dry weight, 4·36 g.; *i.e.* 1·09 g. dry weight each egg. Scyllitol isolated; traces only, insufficient for weighing.

The material used in the above experiments was not very abundant, for it is somewhat difficult to collect, but the results are quite concordant and permit of the conclusion that the scyllitol present in the embryos at the end of development has to be synthesised by them and is not provided in the egg at the beginning. The dogfish is thus in exactly the same position as the chick. In Fig. 1 are shown graphically the relations which have been found to hold in the two kinds of eggs. Eastcott [1928] determined the amount of *i*-inositol present in undeveloped hen's eggs by a biological assay dependent on the identification of inositol with Bios I, and, as the diagram shows, her results agree very well with the figures obtained in direct analysis. It is interesting to note that scyllitol itself will not function as Bios I (Eastcott: private communication).

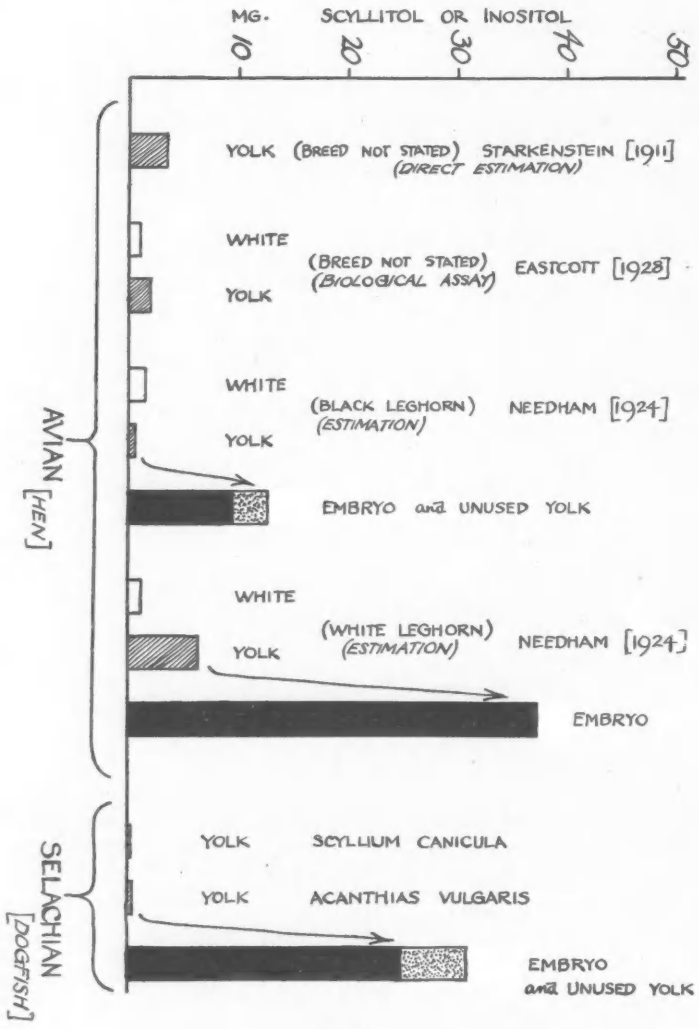
MG. SCYLLITOL OR INOSITOL
10
20
30
40
50
YOLK (BREED NOT STATED) STARKENSTEIN [1911]
(DIRECT ESTIMATION)
WHITE
YOLK (BREED NOT STATED) EASTCOTT [1928]
(BIOLOGICAL ASSAY)
WHITE
YOLK (BLACK LEGHORN) NEEDHAM [1924]
(ESTIMATION)
EMBRYO and UNUSED YOLK
WHITE
YOLK (WHITE LEGHORN) NEEDHAM [1924]
(ESTIMATION)
EMBRYO
AVIAN [HEN]
YOLK SCYLLIUM CANICULA
YOLK ACANTHIAS VULGARIS
EMBRYO
and UNUSED YOLK
SELACHIAN [DOGFISH]

Fig. 1.

One further point which emerges from the data given above may be mentioned. The distribution of solid and water is evidently different in the embryos from what it is in the eggs, and the facts may be written as follows:

		Water	Solid
		g.	g.
Acanthias vulgaris:	Undeveloped eggs	14·2	8·8
	Finished embryos	35·0	6·0

This is another illustration of the rule that in fishes the egg is not provided with sufficient water to make the embryo, but has to absorb it from the environment, and, as it were, to dilute the raw materials with it. No doubt in the case of *Acanthias*, where the eggs remain within the maternal body, the water required is supplied from the maternal blood, but this dogfish stands low in the Ercolani placenta classification, for it has nothing but pits and inequalities on the maternal side, pressed up against the smooth horny egg-case. Little else than water, therefore, probably passes between maternal blood and the eggs.

The present communication adds further to the evidence that cyclose can be synthesised by animal cells. To the two cases of its formation in the egg may be added its formation by polyuric rats on pure diet, and by the plant, as has recently been shown by Eastcott for yeast. It would be interesting to know whether the eggs of lamellibranchiate gastropods contain enough mytilitol for the needs of the embryo or not.

Summary.

The scyllitol of the undeveloped eggs and fully developed embryos of the selachian fish, *Acanthias vulgaris*, was investigated. It was found that just as the chick has to synthesise during its ontogeny 90 % of the inositol with which it hatches, so the dogfish has to synthesise 90 % of its scyllitol. The cycloses would therefore seem not to be provided in the eggs of birds or fishes by the maternal organism.

The writer's thanks are due to Mr Richard Elmhirst and the staff of the Millport Marine Biological Laboratory whose kind assistance and co-operation made the work possible. He is also indebted to the Government Grant Committee of the Royal Society for a grant which covered the cost of these researches.

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XL. NOTE ON ANAEROBIOSIS AND THE USE OF ALKALINE SOLUTIONS OF PYROGALLOL.

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Effective precautions are usually taken to remove poisonous impurities from hydrogen used in securing anaerobiosis. It is difficult to find in bacteriological literature references to the need for recognising that a poisonous gas, carbon monoxide, is evolved when atmospheric oxygen is absorbed by alkaline solutions of pyrogallol. Wright [1928], following Burrows [1917, 1921] in his method of preparing pure nitrogen from commercial cylinder nitrogen, took precautions to ensure that no carbon monoxide, resulting from the small amount of oxygen present, should pass over into the anaerobic chamber.

The author, in conjunction with Drakeley [1925, 1929], has shown that all alkaline solutions of pyrogallol, of strengths likely to be used in practice, evolve carbon monoxide when oxygen of high purity is absorbed. With cylinder oxygen of about 98 % purity, amounts from 0·1 % up to practically 10 % of carbon monoxide may be formed, and absence of agitation of the absorbent solution favours the production of larger amounts of carbon monoxide than those formed when the solution is vigorously shaken. Oxygen of lower purity, such as atmospheric oxygen, liberated carbon monoxide in all cases investigated, though the percentage evolution was smaller.

These findings have a bacteriological import, since the conditions under which alkaline solutions of pyrogallol are used in bacteriology do not usually favour agitation of the solution during absorption of oxygen. A similar remark may be made with reference to some pomological investigations, as, for example, examination of the course of ripening of fruits in the absence of oxygen.

It was also shown that all alkaline solutions of pyrogallol absorb oxygen, though with varying degrees of efficiency. There appears consequently to be no warrant for the practice of some authors of specifying with exactness a particular concentration of alkali, while leaving vague the amount of pyrogallol.

The method of Rockwell, as quoted by Stitt [1927], is of much interest. Rockwell calls for the use of sodium bicarbonate solution with addition of sodium dihydrogen phosphate, according to the formula:

Sodium bicarbonate	50 g.
Sodium dihydrogen phosphate	0·75 g.
Water	500 cc.

"Keep tightly corked."

In use, "0·5 g. pyrogallic acid and 1 cc. of the charged alkali solution" are mixed *in situ* (culture tube).

This might seem to be an attempt to avoid production of carbon monoxide, but it appears to be an omnibus method for producing carbon dioxide in replacement of the absorbed oxygen. As such, its chemistry is complicated. A qualitative study of a solution prepared according to Rockwell's direction was made. It was found that at room temperature a solution of pyrogallol in sodium bicarbonate solution did not appreciably absorb oxygen whether the acid phosphate were present or not. The amount of bicarbonate specified did not entirely dissolve. A roughly quantitative evolution of carbon dioxide took place when the acid phosphate was added to the bicarbonate solution. When the solution was warmed in an incubator at 38°, oxygen was slowly absorbed, owing to bicarbonate becoming partly transformed into normal carbonate; carbon dioxide was given off, partly as a product of that transformation, and partly from the interaction of acetic acid (an oxidation product of pyrogallol) with the carbonates.

A quantitative study was attempted, though, owing to the incomplete solubility of the components at room temperature, it was thought desirable to vary the quantities. 50 g. of pyrogallol were dissolved in 50 cc. of solution prepared according to the above formula, the solution being then made up to 200 cc. with access of air. After standing overnight at room temperature in a corked bottle, the solution was transferred to a Hempel pipette: thus free carbon dioxide was lost. The solution was treated with four successive amounts of commercial cylinder oxygen of about 98·5 % purity and it was allowed to stand in each case without agitation for periods of 24 or 72 hours in a thermostatically controlled water-bath at 38°. Results of analyses of the residual gases are given in the table.

No. of exp.	Volume of cylinder oxygen cc.	Hours in thermostat (approx.)	Percentage of unabsorbed oxygen	Percentage of carbon dioxide	Percentage of carbon monoxide
1	96·0	72	0·05	64	0·55
2	94·7	24	1·4	36	0·7
3	98·0	24	1·4	1·4	0·8
4	100·0	24	3·7	49	1·0

The percentage of carbon dioxide was variable, and depended largely upon the length of time which had elapsed between the moment the pipette was removed from the thermostat and the time analysis was begun. In Exp. 3 the pipette was allowed to stand 24 hours at room temperature before making analysis of the gases; the carbon dioxide was almost entirely absorbed during cooling.

When measured volumes of air were admitted into the pipette, which was then placed in the thermostat, it was found that the final volume of gas was always greater than the initial volume, owing to production of an amount of carbon dioxide greater than the volume of oxygen absorbed. A similar increase of volume was found when the pipette was filled with inert gases

(nitrogen and argon) left from a previous analysis of air. When the pipette was allowed to stand empty of gas, no gas was produced. It was evident that carbon dioxide was produced during the reversible thermal transformation of bicarbonate into carbonate, the acid phosphate taking no part in this production after the initial evolution, of which the effect is lost when the "charged solution" is mixed with pyrogallol before use.

No accurate figures can be given for the percentage of carbon monoxide evolved when this modification of Rockwell's solution was used as absorbent for atmospheric oxygen, owing to the fact that the gases were measured in a simple Hempel burette, and not in the Bone and Wheeler apparatus which was used in the earlier work. In four analyses of air, a small and just detectable amount of carbon monoxide was produced from about 100 cc. of air; since the limit of measurement of the burette readings was 0·05–0·1 cc., this indicated an evolution of carbon monoxide of the order of 0·3 %, calculated on the oxygen absorbed. The result was in agreement with analyses made, using solutions of pyrogallol in caustic potash.

It would seem desirable that the effect of small amounts of carbon monoxide upon the growth of micro-organisms should be investigated, since even such a weakly alkaline solution of pyrogallol as the modification of Rockwell's solution used in this work, is capable of evolving carbon monoxide when the solution is used as an absorbent of oxygen. In conclusion, it may be said that there is little evidence for the idea of combination between pyrogallol and alkalis in aqueous solution: the term "pyrogallate solution" should be abandoned in favour of "alkaline solution of pyrogallol".

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XLI. SEED FATS OF THE UMBELLIFERAE.

II. THE SEED FATS OF SOME CULTIVATED SPECIES.

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Attention has been directed in earlier communications [Hilditch and Jones, 1928; Hilditch, 1928] to the presence of $\Delta^{6:7}$-octadecenoic acid or petroselinic acid in the fatty oil of parsley and other seeds of the Umbelliferae. It was shown in the preceding paper of this series that the seed fats of two native wild species of the order, *Heracleum Sphondylium* and *Angelica sylvestris*, contain in each case about 20 % of this isomeric oleic acid, together with 44–50 % of oleic, 25–33 % linoleic and about 4 % of palmitic acid. Seven other instances of Umbellate seed fats, drawn from common cultivated varieties, have now been examined and petroselinic acid has been found to be present in considerable amount in each case, the proportion varying from 26–60 % of the total mixed fatty acids.

It is therefore possible to state with some confidence that $\Delta^{6:7}$-octadecenoic acid is characteristic of seed fats of the Umbelliferae. The presence of this acid has not been reported, moreover, in fats originating from any other botanical order with the sole exception of the Araliaceae (a closely allied Umbellate family). (It may be mentioned that, since our former communications, a further analysis of the seed fat of *Hedera Helix* has been published by Steger and van Loon [1928], who state that in this case 55 % of the mixed fatty acids consists of petroselinic acid.)

In the present work we have followed our usual procedure as far as possible, in order to obtain a quantitative measure of the various fatty acids present. The mixed fatty acids from the saponified fats were treated with lead acetate in alcoholic solution and a preliminary separation was thus effected into (1) saturated acids, petroselinic acid, and probably a little ordinary oleic acid, and (2) oleic and linoleic acids, possibly accompanied by traces of palmitic and petroselinic acids. The separation of ordinary oleic acid from petroselinic acid by this method is not likely to be completely quantitative, and therefore to that extent the results of the work must be considered of an approximate nature. The separation is, however, sufficiently marked to permit a reasonably accurate estimate of the content of petroselinic acid in the various fats to be obtained.

Each group of acids was then converted into methyl esters and the latter submitted to fractional distillation at a very low pressure.

Another complication, from the point of view of quantitative work, was the presence of non-fatty matter (frequently of a resinous character) in many of the ester fractions; this rendered the interpretation of the saponification equivalents of the latter doubtful in many cases. The same difficulty was encountered in the investigation of *Heracleum* and *Angelica* fats [Hilditch and Jones, 1928], and our experience confirms that of other workers who have noted that Umbelliferous seed fats are prone to be accompanied by unusually large amounts of non-fatty material both in the form of essential oils and of resinous compounds.

Although as much volatile essential oil as possible was removed by heating each fat in a vacuum prior to its hydrolysis, one or two instances were met with (notably, the seed fats of fennel and carrot) in which there remained some volatile compound of an unsaponifiable nature which eventually came over in the more volatile fractions of the distilled methyl esters.

Again, the saponification equivalents of the main ester fractions from certain of the fats (notably celery and parsnip) were sometimes erratic, apparently owing to small proportions of resinous (? lactonic) compounds which tended to distil along with the methyl esters.

The residues from the methyl ester distillations of course contained comparatively large amounts of non-volatile unsaponifiable matter, which, especially by reason of its dark colour, made the determination of mean saponification equivalents of these fractions extremely difficult.

Qualitative examination of the fatty acids present in the methyl ester fractions obtained indicated in all cases that the acids present were only palmitic, petroselinic, oleic and linoleic[1]; no saturated acid lower than palmitic was detected, and the hexabromide test failed to disclose the presence of any linolenic acid in any of the specimens examined. Consequently the numerical calculations have been based wherever possible on the observed iodine values rather than the equivalents, except that in those fractions containing large amounts of unsaponifiable matter (that is, the residual non-volatile fractions and certain of the lowest boiling fractions in the cases of fennel, carrot and caraway) the proportion of unsaponifiable matter has been determined from the saponification equivalent figures.

Experimental.

The seed fats investigated were extracted by means of carbon tetrachloride from the ground dried seeds. We are much indebted to Mr T. E. Miln, of Messrs Gartons Ltd., who very kindly procured for us supplies of the various

[1] The methods employed in these investigations have not disclosed up to the present the occurrence of a diethylenic acid structurally related to $\Delta^{6:7}$-octadecenoic acid rather than to $\Delta^{9:10}$-octadecenoic acid; but the possibility of the existence of such an acid should not be overlooked.

seeds and ensured that the material handled was of definite botanical origin. The crude seed fats obtained by the solvent extraction (in amounts varying from 350–950 g.) had the characteristics shown in the following table.

Table I.

		Characteristics of seed fat					
						Unsaponifiable matter	
Species	Common name	Fat content of seed %	Saponification equivalent	Acidity (as % oleic acid)	I.V.	Non-volatile	Volatile (up to 150°/1 mm.)
Foeniculum capillaceum Gilib.	Fennel	13·3	309·4	16·9	87·9	11·3	5·0
Daucus Carota Linn.	Carrot	15·9	307·7	8·0	106·4	13·25	0·9
Coriandrum sativum Linn.	Coriander	8·8	289·6	28·6	89·5	9·27	1·1
Apium graveolens Linn.	Celery	14·4	291·8	5·7	129·1	2·9	15·2
Pastinaca sativa Linn.	Parsnip	17·3	272·1	12·9	92·7	2·6	—
Chaerophyllum sativum Gartn.	Chervil	11·3	295·3	1·7	139·4	3·3	—
Carum Carvi Linn.	Caraway	9·0	300·9	9·9	112·1	0·9	13·9

The volatile essential oils removed by vacuum treatment of the heated crude fats were not further investigated, since they had no bearing on the immediate purpose of the research. The non-volatile unsaponifiable matter was not removed from the fatty acids prior to further treatment, but was left to accumulate in the residues from the ester distillations.

The crude seed fats (after removal of volatile matter as described) were saponified with excess of alcoholic potash, and the mixed crude fatty acids isolated and dried.

The fatty acids (1 part) were dissolved in boiling 95 % alcohol (5 parts) and mixed with a boiling solution of lead acetate (0·7 part) in 95 % alcohol (5 parts). After boiling for a few minutes the mixture was set aside overnight at room temperature and the separated lead salts were removed by filtration next morning. The insoluble lead salts were recrystallised from a volume of 95 % alcohol equal to that employed in the original separation, and separated by filtration after standing at room temperature overnight. The recrystallised insoluble lead salts were converted back to the fatty acids, and are referred to below as "solid" acids. The mother liquors from the recrystallisation were (except in the case of parsnip seed fat) united with the original alcoholic filtrates, and the recovered acids therefrom are denoted as "liquid" acids. In the case of the parsnip fatty acids the "liquid" acids from the two alcoholic filtrates were worked up separately.

The yields of the two groups of acids from the various seed fats are shown in Table II.

Each group of fatty acids obtained (1 part) was separately dissolved in methyl alcohol (2 parts) and heated under reflux in presence of 2 or 3 % of sulphuric acid for 2 hours; this effected almost quantitative conversion into

the corresponding methyl esters which, after removal of most of the excess of methyl alcohol, were taken up in ether and washed with dilute sodium carbonate solution until neutral. The crude neutral methyl esters were heated in a vacuum at about 100° to remove the last traces of solvent and were then fractionally distilled.

Table II.

	"Solid" acids		"Liquid" acids	
Seed fat	%	I.V.	%	I.V.
Fennel	60·2	83·1	39·8	110·2
Carrot	57·1	87·0	42·9	145·5
Coriander	59·6	79·8	40·4	104·7
Celery	54·0	84·8	46·0	119·5
Parsnip	46·2	87·6	21·7	128·2
			32·1	119·0
Chervil	57·5	100·7	42·5	175·7
Caraway	28·7	80·2	71·3	127·5

The results of the methyl ester separations are summed up in the next series of tables, together with the calculated composition of the individual fractions, determined in the majority of cases (as already stated) from the iodine values in conjunction with the data obtained by qualitative examination of individual ester fractions.

For those fractions which contained notable amounts of unsaponifiable matter it was essential to utilise in addition the saponification equivalents: in these instances the soap solutions from the saponified esters were thoroughly extracted with ether, and the saponification equivalent and iodine value of the recovered fatty acids (freed from unsaponifiable matter) were then determined. These values calculated back to the corresponding methyl esters are (when necessary) inserted in the tables between brackets.

For purposes of identification, individual ester fractions were hydrolysed and the resulting soaps oxidised by means of dilute ice-cold alkaline permanganate solution according to the directions of Lapworth and Mottram [1925]. The liberated mixture of dihydroxy- and tetrahydroxy-stearic acids was boiled with light petroleum (B.P. 40–50°) to effect the removal of any saturated fatty acids; the latter (only palmitic acid was detected) were purified by crystallisation from aqueous alcohol. The crude hydroxystearic acids were boiled with water in order to remove any sativic acid and then crystallised from ethyl acetate.

Petroselinic acid was readily identified in the form of the corresponding 6 : 7-dihydroxystearic acid, which melts at 121–122°. The 9 : 10-dihydroxy-acid from ordinary oleic acid, which melts at 130°, was used to define the presence of $\Delta^{9:10}$-oleic acid. In all cases the identity of the compounds was checked by determination of mixed melting point with authentic specimens of the respective acids.

In several cases a dihydroxystearic acid (or quite possibly an equilibrium mixture of acids) was encountered which melted consistently at 113–115°.

This substance was only met in oxidation products from the "liquid" esters and it is possible that it indicates the presence, in the acids with soluble lead salts, of a certain amount of petroselinic acid; alternatively, it must be borne in mind that there is some possibility that a linoleic acid corresponding with petroselinic acid may be present in fats of this type. If such an acid exists, the means at present employed in the isolation of petroselinic acid are insufficient to lead to its separation.

We may now proceed to tabulate the analytical data which we have obtained for the seven seed fats investigated.

Table III. *Fennel.*

Methyl esters of "solid" acids.

					Estimated composition		
No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Petroselinate	Unsaponifiable
S 1	6·5	129/144°	284·8	68·9	1·3	5·2	—
S 2	25·8	144/147	294·5	72·9	3·9	21·9	—
S 3	75·9	147/153	298·3	84·5	—	75·9	—
S 4	7·6	Residue	441·7 (345·7)	89·7	—	5·1	2·5
	115·8				5·2	108·1	2·5
				% as esters	4·4	93·4	2·2
				% as acids	4·4	93·3	2·3

Methyl esters of "liquid" acids.

					Palmitate	Oleate	Linoleate	Unsap.
L 1	6·5	80/142°	343·7 (279·5)	71·7	1·4	2·3	1·6	1·2
L 2	5·0	142/145	300·7 (283·7)	102·8	0·5	2·5	1·7	0·3
L 3	43·8	145/147	294·2	120·5	—	26·3	17·5	—
L 4	16·1	Residue	560·6 (345·9)	97·8	—	5·2	3·3	7·6
	71·4				1·9	36·3	24·1	9·1
				% as esters	2·6	50·9	33·7	12·8
				% as acids	2·6	50·6	33·5	13·3

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 60–61° (*ca.* 20 % of fraction); 6 : 7-dihydroxystearic, M.P. 121°
S 3	6 : 7-Dihydroxystearic, M.P. 120–121°, and a trace of saturated acid
L 3	Dihydroxystearic mixture (?), M.P. 113–115°

Estimated composition of mixed fatty acids.

	"Solid" % 60·2	"Liquid" % 39·8	Total	Percentage (excluding unsaponifiable)
Palmitic	2·6	1·1	3·7	4
Petroselinic	56·2	—	56·2	60
Oleic	—	20·1	20·1	22
Linoleic	—	13·3	13·3	14
Unsaponifiable	1·4	5·3	6·7	—

Table IV. *Carrot.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	5·4	69/138°	291·5	73·2	0·8	4·6	—
S 2	33·1	138/149	293·9	81·6	1·6	31·5	—
S 3	12·4	149/152	295·4	84·6	0·1	12·3	—
S 4	34·1	152/153	296·4	86·0	—	34·1	—
S 5	11·5	Residue	326·1	80·0	—	10·4	1·1
	96·5				2·5	92·9	1·1
				% as esters	2·7	96·2	1·1
				% as acids	2·7	96·2	1·1

Methyl esters of "liquid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Oleate	Linoleate	Unsap.
L 1	7·1	118/149°	624·3 (282·0)	154·9 (93·1)	1·1	0·7	1·4	3·9
L 2	6·9	149/154	330·9 (298·4)	123·1 (103·6)	1·7	1·6	2·9	0·7
L 3	10·5	154/156	303·8	138·2	—	4·2	6·3	—
L 4	28·8	156/160	298·8	141·9	—	10·2	18·6	—
L 5	13·5	Residue	373·1	91·4	—	3·8	6·9	2·8
	66·8				2·8	20·5	36·1	7·4
				% as esters	4·1	30·8	54·1	11·0
				% as acids	4·1	30·6	53·8	11·5

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 63·5° (*ca.* 20 % of fraction); 6 : 7-dihydroxystearic, M.P. 120–121°
S 4	6 : 7-Dihydroxystearic, M.P. 121–121·5°
L 4	Dihydroxystearic mixture (?), M.P. 113–115°

Estimated composition of mixed fatty acids.

	"Solid" % 57·1	"Liquid" % 42·9	Total	Percentage (excluding unsaponifiable)
Palmitic	1·5	1·8	3·3	4
Petroselinic	54·9	—	54·9	58
Oleic	—	13·1	13·1	14
Linoleic	—	23·1	23·1	24
Unsaponifiable	0·7	4·9	5·6	—

Table V. *Coriander.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	8·3	110/147°	278·8	54·0	3·1	5·2	—
S 2	7·7	147/152	283·3	61·4	2·2	5·5	—
S 3	81·6	145/146	291·0	79·2	6·2	75·4	—
S 4	15·6	Residue	571·6	78·5	—	8·1	7·5
	113·2				11·5	94·2	7·5
				% as esters	10·2	83·2	6·6
				% as acids	10·1	83·0	6·9

Table V (*cont.*)

Methyl esters of "liquid" acids.

					Estimated composition			
					Palmitate	Oleate	Linoleate	Unsap.
L 1	6·2	70/140°	291·4	71·7	1·9	3·5	0·8	—
L 2	51·8	140/143	293·4	102·1	—	42·1	9·7	—
L 3	12·0	Residue	473·3	89·4	—	6·1	1·4	4·5
	70·0				1·9	51·7	11·9	4·5
				% as esters	2·6	73·9	17·1	6·4
				% as acids	2·6	73·7	17·0	6·7

Fraction	Fatty acids identified
S 1	Palmitic acid, M.P. 57–59° (*ca.* 15 % of fraction); 6 : 7-dihydroxystearic, M.P. 119–120°
S 2 and S 3	6 : 7-Dihydroxystearic, M.P. 120–121°, with traces of saturated acids
L 2	9 : 10-Dihydroxystearic, M.P. 128–129°, with some tetrahydroxystearic, M.P. 171–172°

Estimated composition of mixed fatty acids.

	"Solid" % 59·6	"Liquid" % 40·4	Total	Percentage (excluding unsaponifiable)
Palmitic	6·0	1·1	7·1	8
Petroselinic	49·5	—	49·5	53
Oleic	—	29·7	29·7	32
Linoleic	—	6·9	6·9	7
Unsaponifiable	4·1	2·7	6·8	—

Table VI. *Celery.*

Methyl esters of "solid" acids.

					Estimated composition			
No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Petroselinate	Linoleate	Unsaponifiable
S 1	4·2	85/143°	282·0	67·8	0·9	3·3	—	—
S 2	21·7	138/142	286·4	76·8	2·3	19·4	—	—
S 3	19·4	143/147	289·3	88·2	—	18·9	0·5	—
S 4	10·7	148/150	289·9	88·6	—	10·3	0·4	—
S 5	7·9	Residue	309·2	78·8	—	7·3	—	0·6
	63·9				3·2	59·2	0·9	0·6
				% as esters	4·9	92·7	1·4	1·0
				% as acids	4·9	92·7	1·4	1·0

Methyl esters of "liquid" acids.

					Palmitate	Oleate	Linoleate	Unsap.
L 1	4·2	86/155°	277·9	105·2	0·6	2·1	1·5	—
L 2	28·6	155/157	276·8	123·1	—	16·3	12·3	—
L 3	4·4	157/158	288·4	121·3	—	2·6	1·8	—
L 4	9·5	Residue	279·4	101·6	—	4·7	3·3	1·5
	46·7				0·6	25·7	18·9	1·5
				% as esters	1·3	55·0	40·4	3·3
				% as acids	1·3	54·9	40·3	3·5

Fraction	Fatty acids identified
S 2	Palmitic, M.P. 58–59°; 6 : 7-dihydroxystearic, M.P. 120–121°
S 3	6 : 7-Dihydroxystearic, M.P. 121–122°
L 2	Dihydroxystearic mixture (?), M.P. 113–115°, tetrahydroxsytearic, M.P. 170–171·5°

Table VI (*cont.*)

Estimated composition of mixed fatty acids.

	"Solid" % 54·0	"Liquid" % 46·0	Total	Percentage (excluding unsaponifiable)
Palmitic	2·6	0·6	3·2	3
Petroselinic	50·1	—	50·1	51
Oleic	—	25·3	25·3	26
Linoleic	0·7	18·5	19·2	20
Unsaponifiable	0·6	1·6	2·2	—

Table VII. *Parsnip.*

Methyl esters of "solid" acids.

					Estimated composition		
No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Palmitate	Petroselinate	Unsaponifiable
S 1	5·9	158/160°	281·1	79·9	0·4	5·5	—
S 2	18·5	160/161	285·0	85·5	—	18·5	—
S 3	4·4	Residue	350·0	79·4	—	4·1	0·3
	28·8				0·4	28·1	0·3
				% as esters	1·4	97·5	1·1
				% as acids	1·4	97·5	1·1

Methyl esters of "liquid" acids L 1

(from first crystallisation of lead salts).

					Oleate	Linoleate	Unsaponifiable
L 1/1	3·1	119/157°	291·4	130·6	1·5	1·6	—
L 1/2	4·6	157/159	294·5	127·6	2·4	2·2	—
L 1/3	4·8	Residue	300·5	111·2	2·2	2·0	0·6
	12·5				6·1	5·8	0·6
				% as esters	48·5	46·5	5·0
				% as acids	48·4	46·4	5·2

Methyl esters of "liquid" acids L 2

(from recrystallisation of lead salts).

					Palmitate	Oleate	Linoleate
L 2/1	4·0	108/154°	289·8	109·2	0·2	2·5	1·3
L 2/2	11·2	154/155	297·2	116·0	—	7·3	3·9
L 2/3	4·4	Residue	299·4	110·3	—	3·2	1·2
	19·6				0·2	13·0	6·4
				% as esters	1·2	66·0	32·8
				% as acids	1·2	66·0	32·8

Fraction	Fatty acids identified
S 2	6 : 7-Dihydroxystearic, M.P. 122°, with trace saturated acid
L 1/2	9 : 10-Dihydroxystearic, M.P. 129–130°
L 2/2	Dihydroxystearic mixture (?), M.P. 113–114°

Estimated composition of mixed fatty acids.

		"Liquid"			
	"Solid" % 46·2	L 1 % 21·7	L 2 % 32·1	Total	Percentage (excluding unsaponifiable)
Palmitic	0·7	—	0·4	1·1	1
Petroselinic	45·0	—	—	45·0	46
Oleic	—	10·5	21·2	31·7	32
Linoleic	—	10·1	10·5	20·6	21
Unsaponifiable	0·5	1·1	—	1·6	—

Table VIII. *Chervil.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Linoleate	Unsaponifiable
S 1	3·4	90/141°	282·5	91·3	0·5	2·2	0·7	—
S 2	42·1	141/145	283·6	93·6	4·8	28·8	8·5	—
S 3	10·1	145/149	290·5	105·7	—	7·8	2·3	—
S 4	7·3	Residue	313·6	110·3	—	5·3	1·6	0·4
	62·9				5·3	44·1	13·1	0·4
				% as esters	8·4	70·1	20·8	0·7
				% as acids	8·4	70·1	20·8	0·7

Methyl esters of "liquid" acids.

					Palmitate	Oleate	Linoleate	Unsap.
L 1	5·7	115/154°	285·9	153·5	0·6	0·1	5·0	—
L 2	68·3	154/155	298·5	172·3	—	0·4	67·9	—
L 3	3·8	155/157	297·7	170·5	—	0·1	3·7	—
L 4	10·4	Residue	292·4	135·7	—	0·2	8·1	2·1
	88·2				0·6	0·8	84·7	2·1
				% as esters	0·7	0·8	96·1	2·4
				% as acids	0·7	0·8	95·9	2·6

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 57–59°; 6 : 7-dihydroxystearic, M.P. 120–121°
S 2	Palmitic, M.P. 60–61°; 6 : 7-dihydroxystearic, M.P. 121–122°
L 2	Dihydroxystearic mixture (?), M.P. 113–114°, tetrahydroxystearic, M.P. 169–170°, with traces of saturated acid (M.P. 50–52°) and water-soluble tetrahydroxystearic acid (M.P. 150°)

No linolenic acid was present (absence of insoluble hexabromides)

Estimated composition of mixed fatty acids.

	"Solid" % 57·5	"Liquid" % 42·5	Total	Percentage (excluding unsaponifiable)
Palmitic	4·8	0·3	5·1	5
Petroselinic	40·3	—	40·3	41
Oleic	—	0·3	0·3	0·5
Linoleic	12·0	40·8	52·8	53·5
Unsaponifiable	0·4	1·1	1·5	—

Table IX. *Caraway.*

Methyl esters of "solid" acids.

No.	g.	B.P./1 mm.	Sap. equiv.	I.V.	Estimated composition: Palmitate	Petroselinate	Unsaponifiable
S 1	12·5	123/130°	287·2	66·7	2·8	9·7	—
S 2	13·6	130/132	289·0	79·8	0·9	12·7	—
S 3	14·5	132/133	296·1	84·9	0·2	14·3	—
S 4	4·7	Residue	397·1 (355·6)	64·2 (63·9)	—	3·5	1·2
	45·3				3·9	40·2	1·2
				% as esters	8·6	88·7	2·7
				% as acids	8·6	88·6	2·8

Table IX (*cont.*)

Methyl esters of "liquid" acids.

					Estimated composition			
					Palmitate	Oleate	Linoleate	Unsap.
L 1	3·9	100/142°	310·7 (293·9)	114·6 (114·0)	0·3	1·9	1·5	0·2
L 2	18·9	142/144	295·9	112·0	—	13·2	5·7	—
L 3	49·3	144/146	298·2	123·7	—	27·9	21·4	—
L 4	23·7	146/148	296·0	129·4	—	11·8	11·9	—
L 5	13·8	148/149	295·9	127·3	—	7·2	6·6	—
L 6	15·6	Residue	469·3 (336·1)	122·1 (77·3)	—	5·1	4·7	5·8
	125·2				0·3	67·1	51·8	6·0
				% as esters	0·2	53·6	41·4	4·8
				% as acids	0·2	53·5	41·3	5·0

Fraction	Fatty acids identified
S 1	Palmitic, M.P. 61–62°; 6 : 7-dihydroxystearic, M.P. 121–122°
S 3	6 : 7-Dihydroxystearic, M.P. 120–121°
L 4	Mixture of dihydroxystearic acids

Estimated composition of mixed fatty acids.

	"Solid" % 28·7	"Liquid" % 71·3	Total	Percentage (excluding unsaponifiable)
Palmitic	2·5	0·1	2·6	3
Petroselinic	25·4	—	25·4	26
Oleic	—	38·2	38·2	40
Linoleic	—	29·4	29·4	31
Unsaponifiable	0·8	3·6	4·4	—

Discussion of results.

Inspection of the above tables shows that, whilst the quantitative interpretation of the results cannot reach the order of accuracy obtainable by the fractionation method in the absence of volatile resinous and unsaponifiable matter, the latter is concentrated for the most part in the methyl esters of those acids, the lead salts of which were soluble in alcohol. The analyses of the methyl esters derived from the insoluble lead salts are comparatively straightforward, and qualitative tests led to the ready identification of petroselinic acid, in the form of the characteristic 6 : 7-dihydroxystearic acid, and of palmitic acid. Consequently the figures quoted for the percentages of petroselinic acid are quite definite, and provide the necessary data for the main object of the investigation.

It is convenient to summarise the final results for the composition of the mixed fatty acids of each of the seven seed fats at this point.

Table X. *Percentage composition of fatty acids.*

	Palmitic	Petroselinic	Oleic	Linoleic
Fennel	4	60	22	14
Carrot	4	58	14	24
Coriander	8	53	32	7
Celery	3	51	26	20
Parsnip	1	46	32	21
Chervil	5	41	0·5	53·5
Caraway	3	26	40	31

Quite evidently, petroselinic acid occurs in abundance in each of the seed fats now examined. The proportion in each case varies, however, somewhat widely, and whilst, on the one hand, the lowest figure (26 % in the case of caraway seed fat) is higher than that in *Heracleum* or *Angelica* (20 % [Hilditch and Jones, 1928]), the highest values now recorded (58 % in carrot seed fat and 60 % in fennel seed fat) are much lower than the corresponding value for parsley fat (76 % [Hilditch and Jones, 1927]). It is, of course, impossible to make any speculations as to the cause of the varying proportions of the acid in different genera, or even to suggest why Umbelliferae and some related orders of plants should elaborate an isomeric form in addition to the usual oleic acid. The variability in composition may, however, be contrasted with the case of the Palmae, in which genera so diverse as *Cocos nucifera*, *Elaeis guineensis* and *Attalea Cohune* contain not only the same mixture of fatty acids but (within 2 or 3 %) the same proportions of the predominating components, lauric, myristic and palmitic acids [Hilditch, 1928].

An attempt was made in the case of fennel, carrot and caraway seeds to determine whether the composition of the endosperm fat differed materially from that of the fatty oil present in the vittae. The dried seeds were ground up and then exposed to a current of air in such a way that the lighter fragments of the mericarp structure were displaced and the heavier particles collected. The results, however, were quite inconclusive, for on separating the fatty acids obtained from the fat in the concentrated heavy material by the lead salt method, the data observed were as follows:

Table XI.

	Fennel	Carrot	Caraway
Whole seed:			
% Fatty oil	13	16	9
"Solid" fatty acids: % of total acids	60	57	29
Iodine value	83·1	87·0	80·2
Separated seed:			
Percentage of whole seed	74	84	93
% Fatty oil	21	15	15
"Solid" fatty acids: % of total acids	63	47	19
Iodine value	81·2	82·5	71·9

So far as can be judged from this experiment, therefore, the variation in percentage of petroselinic acid does not seem to be connected with varying proportions of vittae fat and true seed fat in the different seeds.

The amount of palmitic acid, the only saturated acid present in these seed fats, is comparatively small and constant, the average figure being in the neighbourhood of 3–4 % of the total fatty acids.

Oleic and linoleic acids, which make up the remainder of the total fatty acids, are not present in constant relative proportions; but in the cases of the seed fats of fennel, carrot, celery, parsnip, and caraway the amount of oleic acid present is from 40–60 % of the combined oleic and linoleic acids, and in several cases the proportions are of much the same order.

The oleic and linoleic acid contents of chervil seed fat stand apart from the rest, since this portion of the chervil fatty acids was found to consist almost exclusively of linoleic acid; the tetrahydroxystearic acid of melting-point 169–170° was obtained in quantity from this acid, which therefore would appear to consist largely of the usual $\Delta^{9:10, 12:13}$-linoleic acid.

The low linoleic acid content of coriander seed fat may well be due to accidental circumstances, the seed in this case having deteriorated somewhat during storage; and therefore we do not attach too much significance to this particular figure.

Summary.

Seven further seed fats from cultivated species of the order Umbelliferae have been examined, and it has been found that $\Delta^{6:7}$-octadecenoic (petroselinic) acid is present in quantity in all cases. The view that the presence of this acid is characteristic of Umbellate seed fats is thus strengthened.

The other components of the mixed fatty acids are palmitic acid in small quantity (1–8 %), together with $\Delta^{9:10}$-octadecenoic (ordinary oleic) acid and linoleic acid in somewhat varying proportions.

The fatty oils are accompanied in all cases by considerable amounts of unsaponifiable and resinous matter, which renders the quantitative investigation somewhat less certain than in other cases in which it has been applied.

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XLII. THE DISTRIBUTION OF REDUCING SUBSTANCES BETWEEN PLASMA AND CORPUSCLES; A COMPARISON OF VARIOUS BLOOD-SUGAR METHODS.

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The distribution of "sugar" between plasma and corpuscles has been the subject of much discussion, and the results obtained by different workers have shown wide variation. We shall not attempt to survey the literature here; a summary is given in a paper by Wiechmann [1924]. In view of the work of de Wesselow [1919] it seemed probable that the variation might be, in part, explained by differences in the methods of blood-sugar estimation. De Wesselow compared MacLean's method with the Lewis-Benedict method, and found that whereas the discrepancies between the methods on plasma were small, MacLean's method always gave considerably lower figures on corpuscles.

In a preliminary experiment, on oxalated sheep's blood, we made a similar comparison between the methods of MacLean and of Folin and Wu. In addition to the direct determination, the corpuscle-sugar was calculated from the figures for plasma and whole blood, and the haematocrit reading for the corpuscle volume. The results were as follows (mg. per 100 cc.).

	Plasma	Whole blood	Corpuscle deposit	Corpuscles calculated
MacLean	48	30	0	0
Folin-Wu	51	50	35	48

These striking figures led us to undertake a fuller investigation of the distribution of reducing substances between plasma and corpuscles as determined by various methods, but for the main investigation we worked upon fresh human venous blood.

Experimental.

The subjects of our experiments were healthy adults, men and women, sometimes examined fasting, and sometimes after an ordinary meal or after a dose of glucose.

[1] Holding a grant from the Medical Research Council.

[2] Working with grants from the Rosenberg-Stokvis-Bischoffsheim funds.

"Sugar" was determined directly in whole blood, plasma, and corpuscles. In addition, the corpuscle-sugar was calculated from the figures found for whole blood and plasma and the haematocrit reading for the corpuscle volume. As the figures determined directly on the corpuscle deposit were open to objections (they might have been too high as a result of insufficiently close packing of corpuscles, or too low owing to glycolysis during the time of spinning) we have tabulated only the calculated figures for corpuscles.

In the majority of experiments the procedure was as follows. A sample of blood taken by vein puncture was run into a paraffin-coated tube, and a second sample taken immediately after into a tube containing potassium oxalate. The oxalated blood was used for determinations of sugar in whole blood. The sample in the paraffin-coated tube was centrifuged at once for 2–4 minutes at 3000 r.p.m., and the plasma was pipetted off and oxalated. The corpuscle deposit was oxalated and centrifuged for a further 10 minutes. There was very rarely any clotting in the paraffin-lined tube. This procedure is indicated in the tables as "paraffin-tube technique." In oxalating the samples of whole blood, and of plasma and corpuscles after separation, care was taken to avoid an excess of oxalate—the amount present was approximately 0·15 to 0·3 %.

In a few experiments the whole sample of blood was defibrinated, and no anticoagulant used at any stage; in a few others the whole sample was oxalated.

We were able to complete the measurement of our samples into the appropriate diluting fluids in less than half an hour from the time of puncture, even when many methods were used. Thus there is little risk of error due to glycolysis.

The methods we have used are:

(1) MacLean's method [1919];
(2) the Folin-Wu method [1920];
(3) the Shaffer-Hartmann method as modified by Somogyi [1926];
(4) the method of Hagedorn and Jensen as originally described, using zinc hydroxide filtrates [1923];
(5) the method of Hagedorn and Jensen applied to tungstic filtrates [Hiller, Linder and Van Slyke, 1925; Hawk and Bergeim, 1926];
(6) the modification of the Folin-Wu method recently introduced by Benedict [1928] in which the sensitiveness of the copper reagent is diminished by addition of alanine, sodium nitrate and $NaHSO_3$. This method is indicated in the tables as the Benedict (1928).

A further note on the preparation of the tungstic filtrates is necessary. The amounts of 10 % sodium tungstate and 2/3 *N* sulphuric acid were adjusted according to the different protein content of plasma, whole blood, and corpuscles. For 1 volume plasma, $\frac{1}{2}$ volume of each of the reagents was used; for 1 volume whole blood, 1 volume of each; and for 1 volume corpuscles, $1\frac{1}{2}$ volumes of each; the total being always made up to 10 volumes. These

tungstic filtrates were used for four different methods—Folin-Wu, Shaffer-Hartmann, Hagedorn and Jensen, and Benedict (1928).

In using the colorimetric methods, a standard of approximately the same strength as the blood filtrate was always used.

All determinations were made in duplicate.

We have checked our technique in all the methods by experiments with glucose solutions and glucose added to blood.

Our results are given in Tables I and II.

Table I. *Comparisons between the MacLean and Folin-Wu methods.*

Date	Subject	Remarks		Reducing substance as glucose mg. per 100 cc. Plasma	Whole blood	Corpuscles	Ratio Plasma "sugar" / Corpuscle "sugar"
24. vii. 28	J.G.	1½ hrs. after lunch.	MacLean	93	78	57	1·63
		Paraffin tube technique	Folin-Wu	102	99	95	1·07
27. vii. 28	J.G.	¾ hr. after lunch.	MacLean	94	81	71	1·32
		P. tube technique	Folin-Wu	112	110	107	1·04
4. ix. 28	J.G.	2 hrs. after lunch.	MacLean	102	89	71	1·43
		P. tube technique	Folin-Wu	106	104	101	1·04
5. ix. 28	J.G.	P. tube technique.	MacLean	94	84	71	1·32
		Fasting	Folin-Wu	106	100	92	1·05
		½ hr. after 50 g.	MacLean	160	140	114	1·40
		glucose	Folin-Wu	167	154	137	1·22
		1 hr. after glucose	MacLean	161	136	104	1·55
			Folin-Wu	166	148	125	1·32
		2 hrs. after glucose	MacLean	92	84	74	1·24
			Folin-Wu	88	93	102	0·86
7. ix. 28	K.	2¾ hrs. after lunch.	MacLean	92	80	61	1·51
		P. tube technique	Folin-Wu	99	97	94	1·05
23. vii. 28	F.K.H.	1 hr. after lunch.	MacLean	117	109	95	1·23
		P. tube technique	Folin-Wu	121	120	118	1·03

SUMMARY OF RESULTS.

The methods first compared were those of MacLean and of Folin and Wu. The discrepancies between the two methods will be considered first, and the other methods then compared with these.

MacLean's method compared with the method of Folin and Wu.

There are sixteen comparisons between these two methods. If discrepancies in which the Folin-Wu figure is higher than the MacLean figure are taken as positive, and the reverse discrepancies as negative, we find:

on plasma, the range of discrepancy is − 4 to + 18, average + 8 mg. per 100 cc.
„ whole blood, „ „ + 9 to + 36, „ + 19 „
„ corpuscles, „ „ + 19 to + 49, „ + 31 „

The range of variation is wide, but in every experiment the same effect is shown to a greater or less degree. The discrepancy between the two methods

Table II. *Distribution of reducing substances between plasma and corpuscles as determined by various blood-sugar methods.*

Date	Subject	Conditions	Methods	Reducing substance as glucose mg. per 100 cc.			Ratio Plasma "sugar"
				Plasma	Whole blood	Corpuscles	Corpuscle "sugar"
17. x. 28	R.J.B.	1½ hrs. after lunch. P. tube technique	MacLean	120	113	105	1·14
			Hagedorn (zinc)	—	110	—	—
			„ (tungstic)	124	140	162	0·76
			Folin-Wu	131	128	124	1·06
			Shaffer-Hartmann	136	135	135	1·01
21. x. 28	F.K.H.	2 hrs. after lunch. Oxalated blood	MacLean	104	83	50	2·08
			Hagedorn (zinc)	99	85	63	1·57
			„ (tungstic)	86	103	130	0·66
			Folin-Wu	109	109	109	1·00
			Shaffer-Hartmann	105	105	105	1·00
24. x. 28	P.	Immediately after tea. P. tube technique	Hagedorn (zinc)	148	133	112	1·23
			„ (tungstic)	163	170	180	0·91
			Folin-Wu	160	158	155	1·03
			Shaffer-Hartmann	158	159	161	0·98
			Benedict (1928)	162	150	132	1·23
26. x. 28	K.	2 hrs. after lunch. P. tube technique	Hagedorn (zinc)	77	71	62	1·24
			„ (tungstic)	89	101	118	0·75
			Folin-Wu	89	87	85	1·05
			Shaffer-Hartmann	84	83	84	1·00
			Benedict (1928)	90	81	68	1·32
29. x. 28	G.A.H.	1½ hrs. after lunch. P. tube technique	MacLean	95	81	62	1·53
			Hagedorn (zinc)	110	94	72	1·53
			„ (tungstic)	106	110	116	0·92
			Folin-Wu	106	101	94	1·11
			Shaffer-Hartmann	110	102	91	1·21
			Benedict (1928)	110	96	77	1·43
1. xi. 28	B.	After lunch. P. tube technique	Hagedorn (zinc)	108	106	103	1·05
			„ (tungstic)	114	131	153	0·75
			Folin-Wu	120	115	109	1·10
			Shaffer-Hartmann	115	115	115	1·00
			Benedict (1928)	118	105	86	1·25
5. xi. 28	K.M.H.	3 hrs. after lunch. P. tube technique	MacLean	137	127	113	1·21
			Hagedorn (zinc)	142	124	98	1·45
7. xi. 28	G.	1½ hrs. after lunch. P. tube technique	MacLean	112	102	90	1·24
			Hagedorn (zinc)	119	107	93	1·28
			„ (tungstic)	141	134	126	1·12
			Folin-Wu	123	119	114	1·08
			Shaffer-Hartmann	120	112	103	1·17
13. xi. 28	F.K.H.	After lunch. P. tube technique	MacLean	99	81	51	1·94
			Hagedorn (zinc)	95	80	55	1·73
			„ (tungstic)	100	110	127	0·78
			Folin-Wu	102	99	94	1·06
			Shaffer-Hartmann	97	97	97	1·00
			Benedict (1928)	105	89	62	1·69
14. xi. 28	R.J.B.	3 hrs. after lunch. Oxalated blood	MacLean	95	85	69	1·38
			Hagedorn (zinc)	98	85	66	1·52
			„ (tungstic)	106	124	156	0·68
			Folin-Wu	109	111	114	0·96
			Shaffer-Hartmann	102	109	120	0·85
			Benedict (1928)	116	113	108	1·07
15. xi. 28	L.R.W.P.	3 hrs. after lunch. Defibrinated blood	MacLean	105	94	80	1·38
			Hagedorn (zinc)	114	104	92	1·24
			„ (tungstic)	119	132	149	0·80
			Folin-Wu	118	120	122	0·97
			Shaffer-Hartmann	116	114	112	1·04
			Benedict (1928)	112	107	101	1·11
7. i. 29	F.K.H.	2 hrs. after lunch. Defibrinated blood	MacLean	82	62	33	2·48
			Shaffer-Hartmann	86	86	86	1·00

is always greater on whole blood than on plasma, and greatest on the corpuscle figures. This was confirmed by the direct determinations on corpuscle deposits, which have not been tabulated.

The Shaffer-Hartmann method as modified by Somogyi.

This method gives figures agreeing well with the Folin-Wu figures, and, like them, showing a small discrepancy with the MacLean results on plasma, and a marked difference on corpuscles. Discrepancies in which the Shaffer-Hartmann figures are higher have been taken as positive, and the reverse discrepancies as negative, in each case.

There are ten comparisons with the Folin-Wu method, and the discrepancies between Shaffer-Hartmann figures and Folin-Wu figures are:

on plasma:	− 7 to + 5,	average − 3	mg. per 100 cc.
„ whole blood:	− 6 to + 7,	„ 0	„
„ corpuscles:	− 9 to + 11,	„ 0	„

There are eight comparisons with MacLean's method, and the discrepancies are:

on plasma:	− 2 to + 15,	average + 7	mg. per 100 cc.
„ whole blood:	+ 10 to + 24,	„ + 20	„
„ corpuscles:	+ 13 to + 55,	„ + 39	„

The method of Hagedorn and Jensen.

The original method, applied to zinc hydroxide filtrates, gives figures which agree fairly well with MacLean's method, and shows definitely lower figures for corpuscles than for plasma. When the ferricyanide reduction method is applied to tungstic acid filtrates, the results indicate a very high corpuscle-sugar—the highest given by any method. This is the only method which indicates a definitely higher sugar value for corpuscles than for plasma. We will summarise these effects by comparing the original Hagedorn and Jensen method with MacLean's method, the Hagedorn and Jensen method (tungstic filtrates) with the Folin-Wu method, and thirdly, comparing the zinc filtrates with the tungstic filtrates.

The original Hagedorn and Jensen method compared with MacLean's method. There are seven comparisons. If the discrepancies in which the Hagedorn and Jensen method gives the higher figure are taken as positive, the results are:

on plasma,	the range of discrepancy is	− 5 to + 15,	average + 4	mg. per 100 cc.
„ whole blood,	„ „	− 3 to + 12,	„ + 4	„
„ corpuscles,	„ „	− 14 to + 13,	„ + 4	„

In most of the determinations the figures given by the two methods agree closely, and, where there is a definite difference, the method of Hagedorn and Jensen gives the higher figure, and the difference is about the same on plasma and corpuscles.

The Hagedorn and Jensen method, applied to tungstic filtrates, compared with the Folin-Wu method. There are ten comparisons, and, taking discrepancies in which the Hagedorn and Jensen figures are higher as positive, we have:

on plasma,	the range of discrepancy is	− 23 to + 18,	average	+ 2	mg. per 100 cc.
„ whole blood,	„ „	− 6 to + 16,	„	+ 11	„
„ corpuscles,	„ „	+ 12 to + 44,	„	+ 30	„

The wide range of discrepancy on plasma is chiefly due to two extreme experiments. In the remaining eight experiments the range is − 7 to + 1 (average − 2).

The method of Hagedorn and Jensen; zinc filtrates and tungstic filtrates. There are ten comparisons. Discrepancies in which the tungstic filtrates give the higher figures are taken as positive.

on plasma,	the range of discrepancy is	− 13 to + 22,	average	+ 6	mg. per 100 cc.
„ whole blood,	„ „	+ 16 to + 39,	„	+ 27	„
„ corpuscles,	„ „	+ 33 to + 90,	„	+ 56	„

Benedict's modification of the Folin-Wu method.

Compared with the original Folin-Wu method Benedict's modification gives slightly lower figures on whole blood. Taking discrepancies in which the Folin-Wu method gives higher figures as positive, we have:

on plasma,	the range of discrepancy is	− 7 to + 6,	average	− 1	mg. per 100 cc.
„ whole blood,	„ „	+ 2 to + 13,	„	+ 8	„
„ corpuscles,	„ „	+ 6 to + 32,	„	+ 20	„

These figures are based on seven comparisons.

The Benedict (1928) method compared with the original Hagedorn and Jensen method. We take this comparison rather than the comparison with MacLean's method, because we have seven experiments in which the Benedict and Hagedorn and Jensen methods are compared, and only four in which there are both MacLean figures and Benedict figures.

If we take discrepancies in which the Benedict figures are higher than the Hagedorn and Jensen figures as positive, we have:

on plasma,	the range of discrepancy is	− 2 to + 14,	average	+ 9	mg. per 100 cc.
„ whole blood,	„ „	− 1 to + 28,	„	+ 10	„
„ corpuscles,	„ „	− 17 to + 42,	„	+ 10	„

The ratio of plasma-"sugar" to corpuscle-"sugar."

It has already been explained that, in the majority of our experiments, the plasma was obtained from a sample of blood to which no anti-coagulant was added until the plasma had been separated. Under these conditions the true ratio of "sugar" in plasma and corpuscles should be obtained. The average ratios of plasma-"sugar" to corpuscle-"sugar" by the various methods (excluding the few experiments on oxalated blood) are as follows:

MacLean	1·48	(average of	16	determinations)	
Hagedorn-Jensen (zinc filtrates)	1·34	(„	8	„	)
Hagedorn-Jensen (tungstic filtrates)	0·85	(„	8	„	)
Folin-Wu	1·07	(„	17	„	)
Shaffer-Hartmann	1·05	(„	9	„	)
Benedict (1928)	1·34	(„	6	„	

The difference between the methods of MacLean and Hagedorn and Jensen (zinc filtrates) may be exaggerated here, because there are several experiments in which only one of these methods was used. If we take the shorter series of experiments in which both methods were applied to the same blood, the average ratios are much closer together (viz. 1·46 and 1·45). There is considerable variation of the ratio in all the three methods which give the higher ratios, and the number of experiments is not large enough to give strictly comparable averages. A consideration of individual experiments brings out the same relations as are shown by the average figures.

It is noteworthy that in the sugar-tolerance curve (5. ix. 28) the ratios by both MacLean's method and the Folin-Wu method rose when the total sugar was high, and fell when the total sugar was low, whereas the discrepancies between the methods remained fairly steady.

To sum up: we have two methods which agree fairly well and indicate a higher sugar value in plasma than in corpuscles, namely MacLean's method and the original Hagedorn and Jensen method. The Benedict (1928) method[1] gives a similar distribution ratio, but this ratio is derived from slightly higher figures for both the plasma and corpuscles. We have two methods which agree very well and indicate an approximately even distribution of "sugar" between plasma and corpuscles—the Folin-Wu and Shaffer-Hartmann methods. Finally, the Hagedorn and Jensen method applied to tungstic filtrates is alone in indicating a higher sugar figure for corpuscles than for plasma, thus differing very greatly from the same method of estimation applied to zinc hydroxide filtrates.

Possible effects due to technique.

Before discussing the significance of these results we must consider two factors inherent in the technique, which might unequally affect the determinations on plasma, whole blood, and corpuscles.

The first is the possible error introduced by taking an aliquot part of a blood filtrate. The original method of Hagedorn and Jensen is free from this objection, because the protein precipitate is washed and the whole filtrate with washings is used. In the MacLean method of protein precipitation the blood is diluted 1 in 125, so that the volume of the precipitate is negligible. In the tungstic precipitation the conditions are different. The dilution of the blood is 1 in 10, and there is an apparently voluminous protein precipitate which is greater in whole blood than in plasma, and greater still when corpuscles are used. We have observed that there is no volume change on mixing the reagents: the total volume of the mixture, including the precipitate, is 10 cc. for 1 cc. of blood. If the volume of the precipitate is appreciable, the filtrate must be concentrated, and since, when taking an aliquot part, we

[1] Rockwood [1926] has made comparisons between the Folin-Wu method and Benedict's earlier modification of it (not the method we have used). He found that the differences between the original Folin-Wu and the modification were most pronounced in the corpuscle figures.

assume that the fluid volume is still 10 cc., our figures will be too high, the error being greatest when the protein precipitate is greatest. We tested this experimentally as follows. The usual mixtures of plasma, whole blood, or corpuscles with the sodium tungstate and sulphuric acid were made in graduated centrifuge tubes; the protein was centrifuged down and the supernatant liquid removed, and the tubes were dried to constant weight. A known volume of absolute alcohol was pipetted into each tube and the level noted. In this way the solid volume was estimated by displacement; for plasma we found it 0·5 % of the total volume of precipitation mixture, for whole blood 2 %, and for corpuscles 2·5 %. These figures are only approximate, but show that the effect is negligible and that no appreciable error is introduced by taking aliquot parts of the filtrate.

Secondly, in the tungstic precipitation and the MacLean precipitation the reagents are acid, and some hydrogen ions are taken up by the protein. Since there is less protein in the plasma and more in the corpuscles, the acidity of the filtrates from plasma is greater than that of filtrates from whole blood. In our tungstic filtrates from plasma the p_H was approximately 4·8, from the whole blood 5·1, and from corpuscles 5·3. There was thus little difference, and we found that adjustment of the filtrates to approximately p_H 6·0 had no effect on the figures obtained. The copper reduction methods are sensitive to changes in p_H, as has been emphasised by Folin and Svedberg [1926] and Somogyi [1926], but in our filtrates the difference was too small to introduce any error. Similarly, in the MacLean method, the differences in acidity of filtrates are too small to affect the results. In the zinc hydroxide filtrates used for the Hagedorn and Jensen method, there is no appreciable difference in p_H between filtrates from plasma, whole blood, and corpuscles; all three are approximately of p_H 6·6.

A non-glucose reducing substance.

We know that all the methods estimate glucose quantitatively, whether in pure solution or added to blood. It is therefore unlikely that the methods which give low figures on corpuscles are failing to estimate glucose, and we are led to postulate the presence of some non-glucose reducing substance, predominantly present in corpuscles, which may account for the discrepancies between methods. The methods giving lower, and therefore probably truer, glucose figures are the methods of MacLean and Hagedorn and Jensen (zinc filtrates). These methods are absolutely different in principle, and yet give fair agreement. But when we apply the ferricyanide reduction to tungstic filtrates we obtain the highest figures of any for corpuscle-sugar. The interfering substances must be present in tungstic filtrates, and either absent from the iron filtrates and zinc filtrates, or present in them in smaller amount. Of the four methods applied to tungstic filtrates, three give high corpuscle figures—Hagedorn and Jensen, Shaffer-Hartmann, and Folin-Wu—and the tungstic precipitation is the only feature common to the three methods. The

Benedict (1928) method is the only method applied to tungstic filtrates which gives a distribution ratio similar to that given by MacLean's method, and the copper reagent used in the Benedict (1928) method is designed to be relatively insensitive, and therefore more selective than the copper reagent of the Folin-Wu method. Benedict claims that his modification gives true sugar figures, but it seems probable that the figures given by his method, though lower than the Folin-Wu figures, are slightly above the true sugar value, since we find that the methods of MacLean and Hagedorn and Jensen (zinc filtrates) give lower figures still.

We have further evidence of the important part played by the method of protein precipitation. We have applied MacLean's method to tungstic filtrates as follows. 2 cc. of a 1 in 10 tungstic filtrate are diluted with 18 cc. of the acid sodium sulphate solution used in MacLean's method: 2 cc. of the copper solution are added, and the estimation carried out in the usual way. (The filtrate taken corresponds to 0·2 cc. blood, *i.e.* 5/4 of the amount in the ordinary MacLean method.) This modification of the method quantitatively determines glucose added to blood. In Table III are given comparisons between the ordinary MacLean method, the MacLean method applied to tungstic filtrates, and the Folin-Wu or Shaffer-Hartmann methods. MacLean's method of estimation gives higher figures on tungstic than on iron filtrates, and the MacLean estimation applied to tungstic filtrates usually agrees with the Folin-Wu or Shaffer-Hartmann method. The distribution ratio given by the MacLean method applied to tungstic filtrates is close to unity, and differs greatly from the ratio given by the ordinary MacLean method.

Table III.

	Methods		Reducing substance as glucose, mg. per 100 cc.		
			Plasma	Whole blood	Corpuscles (directly determined)
I	MacLean	Iron filtrate	—	75	—
	„	Tungstic filtrate	—	83	—
	Folin-Wu	„	—	102	—
II	MacLean	Iron filtrate	120	113	—
	„	Tungstic filtrate	124	123	—
	Folin-Wu	„	131	128	—
III	MacLean	Iron filtrate	—	87	—
	„	Tungstic filtrate	—	94	—
	Folin-Wu	„	—	100	—
IV	MacLean	Iron filtrate	101	84	78
	„	Tungstic filtrate	120	118	109
	Shaffer-Hartmann	„	—	116	115
V	MacLean	Iron filtrate	82	62	—
	„	Tungstic filtrate	91	88	88
	Shaffer-Hartmann	„	86	86	86

We are thus led to the hypothesis that there is some non-glucose reducing substance present in corpuscles. The various methods applied to tungstic filtrates vary in their sensitiveness to this unknown substance, the ferri-

cyanide reduction being the most sensitive, and the Benedict (1928) copper reduction the least.

Direct evidence of a non-glucose reducing substance in corpuscles. In addition to the inference drawn from our comparison of methods, we have direct evidence of a reducing substance other than glucose in tungstic filtrates of whole blood or corpuscle deposits.

If a tungstic filtrate from whole blood is added to the alkaline copper reagent used in the Folin-Wu estimation, and the phosphomolybdate reagent is added at once, in the cold, a definite blue colour develops. This reaction is not given by plasma filtrates, and is given very strongly by corpuscle filtrates. The reaction does not occur if the blood filtrate and phosphomolybdate are mixed in the absence of the copper solution; it is a reduction of the copper reagent, not a direct reduction of the phosphomolybdate reagent.

Colloidal ferric hydroxide filtrates from blood do not give the reaction with the Folin-Wu copper solution, neither do zinc hydroxide filtrates. For the purpose of this test the iron or zinc filtrates were of the same concentration as the tungstic filtrates—10 cc. filtrate corresponding to 1 cc. blood. We have therefore direct evidence of a reducing substance in corpuscles, which passes into tungstic filtrates and not into iron or zinc filtrates.

It is possible to remove glucose from blood by a short incubation with a large excess of yeast, and to precipitate the mixture with tungstic acid [Somogyi, 1927]. Such filtrates from fermented blood still give the reaction with the Folin-Wu copper solution in the cold.

The probable nature of the unknown substance. The idea that non-glucose reducing substances in blood may affect blood-sugar estimations is not new; it is known that some of the methods give a residual reduction after fermentation with yeast. The earlier work on this residual reduction was unsatisfactory, owing to technical difficulties, but these were overcome by the discovery of Hiller, Linder and Van Slyke [1925], that a very short incubation, with excess of yeast, was sufficient to remove glucose. Somogyi, using this principle, has determined the non-fermentable reducing substances in whole blood, plasma and corpuscles, by his modification of the Shaffer-Hartmann method. In his first paper [1927] he gave the figure for whole blood as 27 mg. per 100 cc., but his later technique [1928] gave figures 3 or 4 mg. lower; on this basis we may take the figure for plasma as 8, for whole blood 23, and for corpuscles 40 mg. per 100 cc. Now we have found that MacLean's method gives figures lower than the Shaffer-Hartmann method as modified by Somogyi, and that the discrepancy is for plasma 7, for whole blood 20, and for corpuscles 39 mg. per 100 cc. These are average figures. The agreement between these discrepancies and Somogyi's figures for non-glucose reducing substances is striking, and naturally leads to the suggestion that MacLean's method gives figures very close to the true sugar value.

We must now consider some substances which might be responsible for the residual reduction after fermentation with yeast, and for the discrepancies between methods.

Uric acid, creatine and creatinine may be dismissed, for Hiller, Linder and Van Slyke [1925] have shown that their reduction effect is too small to be of importance, in view of the small amounts present in blood.

Ergothioneine is a reducing substance, present only in corpuscles, and it is capable of affecting blood-sugar methods [Sjollema, 1927]. Hunter [1928] has estimated the amount present in normal human blood, and finds 2–10 mg. per 100 cc. corpuscles, *i.e.* a maximum of 4 or 5 mg. per 100 cc. whole blood. We have made a few determinations of the effect of ergothioneine solutions upon the various blood-sugar methods, and find that 100 mg. ergothioneine is equivalent to 10–60 mg. glucose, according to the method used. In view of the very small amount of ergothioneine in human blood, the effect is negligible.

Glutathione is known to be present in corpuscles, mainly in the reduced form [Hunter and Eagles, 1927; Holden, 1925; Thomson and Voegtlin, 1926; Uyei, 1926]. It is capable of reducing ferricyanide and the Folin-Wu reagents [Sjollema, 1927]. We have found that solutions of reduced glutathione reduce the Folin-Wu copper reagent in the cold; glutathione is therefore probably responsible for this reaction as given by tungstic filtrates of corpuscles.

It is well known that tungstic filtrates from corpuscles give a positive nitroprusside reaction, whereas tungstic filtrates from plasma do not. On the basis of the intensity of the nitroprusside reaction, Hunter and Eagles estimate the glutathione in human blood at 100–120 mg. per 100 cc. corpuscles, *i.e.* 40–50 mg. per 100 cc. blood.

The subject of the quantitative effect of glutathione in the various blood-sugar methods, and, in particular, its behaviour in the various methods of protein precipitation, is still under investigation, and will form the subject of a later paper. It may be said at present that concentrations of reduced glutathione, of the order of 45 mg. per 100 cc., give a significant reduction of all the blood-sugar reagents except that of Benedict (1928).

Significance for general analytical work.

We have shown that differences of the order of 30 mg. per 100 cc. are commonly found, when different methods are applied to the same sample of whole blood. Differences of this order are of importance in clinical work, and it is necessary to interpret results according to the method used. Also our findings give an explanation of some of the varying results obtained by different workers for the distribution of "sugar" between plasma and corpuscles. Further, our results suggest that the choice of method may be of great importance in the determination of "sugar" in tissue extracts or other material in which glutathione and other reducing substances are likely to be present, particularly if the relative amount of glucose is small and concentrated extracts are used (*e.g.* in glycolysis experiments).

SUMMARY.

1. The various methods of blood-sugar estimation give widely different results for the distribution of "sugar" between plasma and corpuscles. The average discrepancies between methods are not great for estimations on plasma, but are considerable for estimations on corpuscles.

2. The methods of MacLean and Hagedorn and Jensen (original) agree fairly well, and give a higher sugar value in plasma than in corpuscles.

3. The methods of Folin-Wu and Shaffer-Hartmann (Somogyi's modification) agree very well, and indicate approximately even distribution of reducing substances between plasma and corpuscles.

4. In comparing the method of MacLean or Hagedorn and Jensen (original), with the method of Folin-Wu or Shaffer-Hartmann, we find that the first pair of methods gives slightly lower figures than the second pair of methods on plasma, and considerably lower figures on corpuscles.

5. Benedict's latest modification of the Folin-Wu method gives ratios of plasma-"sugar" to corpuscle-"sugar" similar to those given by the method of MacLean or the original Hagedorn and Jensen method, but the figures given by the Benedict (1928) method are slightly higher on both plasma and corpuscles.

6. The method of Hagedorn and Jensen applied to tungstic filtrates gives very high corpuscle figures, and is the only method which shows more "sugar" in corpuscles than in plasma. Zinc hydroxide filtrates from whole blood or corpuscles give very much lower figures than tungstic filtrates when estimated by the ferricyanide method.

7. MacLean's method may be applied to tungstic filtrates and the results for corpuscles are much higher, by this modification, than by the original method.

8. We suggest that the discrepancies between blood-sugar methods are due to the presence of a non-glucose reducing substance in corpuscles, which is present in tungstic filtrates, and is either absent from iron and zinc filtrates, or present in them in less amount. We have direct evidence of the presence of such a substance, because tungstic filtrates of whole blood or corpuscles produce a reduction of the Folin-Wu copper reagent in the cold, whereas filtrates from plasma give no such reaction. The reaction is not given by iron filtrates or zinc filtrates.

9. It is suggested that the substances responsible for the discrepancies between methods may also be responsible for the residual reduction after fermentation of blood with yeast.

10. Uric acid, creatine, creatinine, and ergothioneine are dismissed as having no appreciable effect on blood-sugar methods, in the small amounts in which these substances occur in human blood.

11. Glutathione is suggested as having an important effect on blood-sugar methods.

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XLIII. MEMBRANE EQUILIBRIA AND SELECTIVE ABSORPTION.

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Two general theories have been advanced to account for the unequal distribution of inorganic electrolytes on either side of a cell membrane.

According to one theory, the unequal distribution is due to the existence of a cell membrane, which not only prevents the passage of the colloid constituents of the cell, but also has the property of presenting varying resistance to the passage of different inorganic ions. According to the other theory, the cell membrane is capable of preventing the passage of the colloid constituents of the cell, but the unequal distribution of inorganic salts is due to the formation within the cell of relatively undissociated and non-diffusible compounds of the various ions with the cell constituents.

Direct experiments designed to test the former theory are beset with considerable difficulties, since the experiments have to be carried out on living tissues, and any considerable alterations in the surrounding medium are liable to alter the properties of the membrane. On the other hand, many artificial membranes exist which may be used in testing the latter theory, since the only properties which the membrane must have are those of preventing the passage of colloids and of allowing the free passage of inorganic salts.

In previous work [Wright, 1926] an attempt was made to ascertain how far the secretion of high concentrations of calcium in milk from the relatively low concentrations of this element in the blood-plasma could be accounted for on the basis of an unequal distribution caused by the presence in the milk cells of non-diffusible and relatively undissociated protein molecules. For this purpose, solutions of sodium caseinogenate were separated from solutions of calcium salts by a membrane permeable to inorganic ions but impermeable to the caseinogen molecules or ions. It was found that under these conditions the calcium tended to accumulate in high concentration on the protein side of the membrane, the distribution ratios[1] for the calcium varying from 2·2 to as high as 12·8. No attempt was made in the preliminary paper to examine in detail the physical chemistry of the systems under investigation.

[1] Throughout this paper, the term "distribution ratio" indicates, for cations, the ratio of the concentration on the protein side of the membrane to that on the non-protein side, and for anions, the ratio of the concentration on the non-protein side to that on the protein side.

The results appeared, however, to have such important applications in the more general study of the unequal distribution of salts in living tissues, that it was decided to carry out further, and more detailed, experiments. The results of these later experiments, which are described in this paper, show conclusively that (in the systems studied) the observed inequalities in the distribution ratios of the salts can be entirely explained if two factors—the degree of dissociation of the protein salts and the establishment of a Donnan equilibrium—are taken into account.

Technique.

100 cc. of each of the two solutions under investigation were placed on either side of a dialysing vessel, the solutions were allowed to come into equilibrium by dialysing for at least 24 hours, and the contents were then removed and analysed.

The caseinogen used in the experiments was prepared by the method detailed by Van Slyke [1923] which gives a product of remarkably low ash content. The ash in the samples used was less than 0·1 %.

The dialysing vessels were of the type devised by Wright and Rule [1927], cellophane (0·0017 cm. thick) being employed as the membrane. This membrane is impermeable to caseinogen but freely permeable to inorganic salts.

With regard to analysis, calcium was estimated by McCrudden's [1909] method, and chloride by a macro-method based on that of Van Slyke [1924]. Sodium was determined throughout by difference[1]. The hydrogen ion concentration of the solutions was not rigidly controlled, but the solutions were in all cases kept between p_H 6·7 and 7·0. It will be seen from the results that the small variations had no significant effect on the equilibria obtained. Toluene was added in order to prevent bacterial decomposition of the protein.

Results.

Table I shows the results obtained with simple systems containing Na caseinogenate and NaCl. It will be seen that, at equilibrium, the distribution ratios of the sodium are in every case greater than the corresponding ratios of the chloride.

If Donnan's theory holds for such systems, this inequality in the distribution ratios can only be accounted for on the assumption that the Na caseinogenate is not completely dissociated. By calculation from the experimental data, it is found that a percentage dissociation of the caseinogenate of between 62 and 77 % (with a mean value of 68 %) is necessary in order to make the ionic ratios equal. Such a value agrees closely with that obtained from conductivity data by Pauli [1922].

[1] *I.e.* total anions – total calcium, assuming that 5 % caseinogen acts as a 0·025 *N* anion. All calculations in this paper are based on the analytical data, and not on the quantities of electrolytes noted as initially present. The latter are approximate values only.

Table I. *Equilibria in systems containing sodium caseinogenate and sodium chloride.*

Exp. No.	Approximate initial concentrations: Side I Caseinogen %	Side I NaOH N	Side II NaCl N	Concentrations at equilibrium: Sodium I N	Sodium II N	Chloride I N	Chloride II N	Ratios at equilibrium: $\frac{Na_I}{Na_{II}}$	$\frac{Cl_{II}}{Cl_I}$	Percentage dissociation of caseinogenate
1	5·0	0·0250	0·1000	0·0688	0·0526	0·0438	0·0526	1·31	1·20	77
2	5·0	0·0250	0·0500	0·0443	0·0269	0·0193	0·0269	1·65	1·39	67
3	5·0	0·0250	0·0250	0·0337	0·0145	0·0087	0·0145	2·32	1·67	62
4	5·0	0·0250	0·0125	0·0288	0·0088	0·0038	0·0088	3·28	2·31	66
									Mean	68

Table II. *Equilibria in systems containing calcium caseinogenate and calcium chloride.*

Exp. No.	Approximate initial concentrations: Side I Caseinogen %	Side I $Ca(OH)_2$ N	Side II $CaCl_2$ N	Concentrations at equilibrium: Calcium I N	Calcium II N	Chloride I N	Chloride II N	Ratios at equilibrium: $\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$	$\left(\frac{Cl_{II}}{Cl_I}\right)^2$	Percentage dissociation of caseinogenate
5	2·0	0·0100	0·0250	0·0224	0·0132	0·0120	0·0129	1·70	1·07	1·14	29
6	5·0	0·0250	0·0250	0·0362	0·0137	0·0115	0·0137	2·64	1·19	1·41	32
7	5·0	0·0250	0·0125	0·0292	0·0073	0·0056	0·0072	4·00	1·28	1·64	27
8	7·0	0·0350	0·0125	0·0396	0·0077	0·0053	0·0078	5·14	1·47	2·16	33
										Mean	30

Table II shows the results obtained with simple systems containing Ca caseinogenate and $CaCl_2$. The results are similar to those shown in Table I, but the distribution ratios of the calcium and the chloride (the ratio of the latter being squared[1]) show a far greater divergence from one another than do the corresponding sodium and chloride ratios of the previous experiments.

If Donnan's theory holds for the calcium systems, the degree of dissociation of the Ca caseinogenate must be considerably lower than that of the Na caseinogenate: on calculation (by a similar method to that employed above) the percentage dissociation is in fact found to lie between 27 and 33, with a mean value of 30. Such a value agrees well with that obtained from conductivity data by Robertson [1920].

Table III. *Equilibria in simple and in mixed solutions.*

Exp. No.	Approximate initial concentrations: Side I Caseinogen %	Side I NaOH N	Side I $Ca(OH)_2$ N	Side II NaCl N	Side II $CaCl_2$ N	Concentrations at equilibrium: Sodium I N	Sodium II N	Calcium I N	Calcium II N	Chloride I N	Chloride II N	Ratios at equilibrium: $\frac{Na_I}{Na_{II}}$	$\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$
3	5·0	0·025	—	0·025	—	0·0337	0·0145	—	—	0·0087	0·0145	2·32	—	1·67
6	5·0	—	0·025	—	0·025	—	—	0·0362	0·0137	0·0115	0·0137	—	2·64	1·19
9	5·0	0·025	—	—	0·025	0·0142	0·0100	0·0215	0·0035	0·0107	0·0135	1·42	6·15	1·26

In Table III typical results obtained in these simple systems are compared with the results obtained in a mixed system containing Na caseinogenate and $CaCl_2$. It is in this experiment, and in the succeeding experiments, that the very important influence of the degree of dissociation in systems of mixed

[1] To conform to Donnan's equilibrium equation.

electrolytes becomes apparent. It will be seen that the low degree of dissociation of the Ca caseinogenate has caused a preferential absorption of the calcium to the protein side of the membrane, with a consequent high distribution ratio of calcium of 6·15, and lowered distribution ratios of the sodium and chloride of 1·42 and 1·26 respectively.

Table IV. *Equilibria in systems containing sodium caseinogenate, sodium chloride and calcium chloride.*

	Approximate initial concentrations				Concentrations at equilibrium						Ratios at equilibrium		
	Side I		Side II		Sodium		Calcium		Chloride				
Exp. No.	Caseinogen %	NaOH *N*	NaCl *N*	$CaCl_2$ *N*	I *N*	II *N*	I *N*	II *N*	I *N*	II *N*	$\frac{Na_I}{Na_{II}}$	$\frac{Ca_I}{Ca_{II}}$	$\frac{Cl_{II}}{Cl_I}$
1	5·0	0·0250	0·1000	—	0·0688	0·0526	—	—	0·0438	0·0526	1·31	—	1·20
10	5·0	0·0250	0·0875	0·0125	0·0573	0·0487	0·0109	0·0026	0·0432	0·0513	1·17	4·17	1·17
11	5·0	0·0250	0·0750	0·0250	0·0501	0·0453	0·0197	0·0056	0·0448	0·0509	1·10	3·50	1·13
12	5·0	0·0250	0·0625	0·0375	0·0428	0·0403	0·0279	0·0103	0·0457	0·0506	1·06	2·71	1·11
13	5·0	0·0250	0·0500	0·0500	0·0367	0·0340	0·0347	0·0158	0·0464	0·0498	1·07	2·21	1·07
14	5·0	0·0250	0·0375	0·0625	0·0286	0·0276	0·0420	0·0218	0·0456	0·0494	1·03	1·93	1·07

A more extended study of mixed systems of this type is shown in Table IV. In this series, the concentration of the Na caseinogenate and the total concentration of chlorides have been kept constant, but the relative concentrations of NaCl and $CaCl_2$ have been varied. The following are the salient features of these results.

(*a*) As the total calcium in the system is decreased, an increasing proportion of calcium passes through the membrane. This is to be expected, since (owing to its low degree of dissociation) the smaller the ratio of calcium to caseinogen, the greater will be the relative amount of calcium bound by the caseinogen.

(*b*) As the total calcium in the system is increased, the distribution ratios of the sodium and chloride decrease, until, with high concentrations of calcium, the ratios approach unity. If the reversible reaction

$$\text{Na caseinogenate} + CaCl_2 \rightleftharpoons \text{Ca caseinogenate} + 2\,\text{NaCl} \quad \ldots(1)$$

is considered, it will be seen that the addition of $CaCl_2$ should tend to force the reaction from left to right, *i.e.* to form additional Ca caseinogenate and NaCl. Hence with increasing calcium in the system, the proportion of undissociated Ca caseinogenate and of freely diffusible NaCl should both increase, and (if Donnan's theory holds good) the distribution ratios of the sodium and the chloride should approach unity. The experimental results confirm this. Actually the distribution ratio of the sodium should always be slightly higher than that of the chloride, since there must always be a small quantity of undissociated Na caseinogenate in the system (the above equation being reversible). The method of analysis was not sufficiently accurate to reveal such a difference. The difference is, however, seen in the results of Exp. 9 of Table III.

Table V. *Effect of varying concentrations of sodium chloride on the distribution of calcium.*

Exp. No.	Approximate initial concentrations: Side I Caseinogen %	Side I NaOH *N*	Side II $CaCl_2$ *N*	Side II NaCl *N*	Concentrations of calcium at equilibrium: I *N*	II *N*	Ratio at equilibrium $\frac{Ca_I}{Ca_{II}}$
9	5·0	0·025	0·025	—	0·0215	0·0035	6·15
11	5·0	0·025	0·025	0·075	0·0197	0·0056	3·50
15	5·0	0·025	0·025	0·75	0·0144	0·0101	1·42
16	5·0	0·025	0·025	2·5*	0·0152†	0·0118	1·29

* Contained appreciable quantities of calcium as an impurity; this accounts for the high analytical values obtained.

† Volume of solution reduced to 85 cc., *i.e.* it is actually a 5·9 % caseinogen solution.

It has been shown above that the presence of increasing concentrations of $CaCl_2$ causes a shift in the equilibrium in equation (1) from left to right. An attempt was made to cause the reverse reaction, *i.e.* to shift the equilibrium from right to left, by adding large quantities of NaCl to the system. Table V shows that this attempt was successful. As the NaCl in the system was increased (the quantities of $CaCl_2$ and of Na caseinogenate being kept constant), the proportion of calcium passing through to the protein side of the membrane decreased, until, with very high concentrations of NaCl, the distribution ratio of the calcium itself approached unity.

Discussion.

The above results fully confirm the main conclusions of the previous paper [Wright, 1926]. Further, they demonstrate that the unequal distribution of inorganic salts on either side of the membrane can be fully explained if two factors—the degree of dissociation of the protein salt, and the establishment of a Donnan equilibrium—are taken into account.

It should be understood that the systems studied have not been selected for the purpose of illustrating striking distribution ratios: they have been selected primarily in order to elucidate the factors involved in the establishment of the unequal distribution. Nevertheless the results, together with those previously published, demonstrate the remarkable alterations in the distribution of salts on either side of an artificial membrane caused by the presence of a non-diffusible electrolyte (caseinogen). Further, they demonstrate the marked influence of the presence of one ion on the distribution ratios of other ions; for instance, in the influence of calcium on the sodium and chloride ratios in Exps. 9 to 14, and in the influence of sodium chloride on the calcium ratios in the experiments shown in Table V. It should be emphasised that such alterations in the distribution ratios are entirely independent of the nature of the membrane, provided that the latter fulfils the conditions necessary for the establishment of a Donnan equilibrium, *i.e.* impermeability to one ion of the system but complete permeability to all other ions.

In drawing attention to the more general applications of these results in

problems of cellular physiology, it is necessary to recognise that simple equilibria of the type discussed in this paper can only provide a partial explanation of the remarkable inequalities in the distribution of inorganic elements in living tissues. In the tissues, the various constituents are undergoing continuous transformations, which alter not only the internal phases of the cell but also the nature of the cell membrane itself. Recent papers on permeability have in fact tended to stress the importance of the constitution of the cell membrane in accounting for the accumulation of inorganic elements in living tissues; the membrane has been assumed to have the property of presenting varying resistance to the passage of different inorganic ions.

While such an assumption has in many cases received justification, the results detailed in this paper point to a further explanation, based on the degree of dissociation of the non-diffusible (protein) salts of the cell and on the establishment of a Donnan equilibrium, which may also be of considerable importance in accounting for the unequal distribution of inorganic elements in living tissues. Specific examples of the possible applications of these results may be cited (1) in the experiments of Stiles and Kidd [1919] on the absorption of salts by carrot tissues, (2) in the experiments of Hoagland [1923] on the influence of one salt on the distribution of another salt between the plant and its surrounding medium, and (3) in the experiments of Loeb and Nichols [1926, 1927] on the diffusibility of the calcium of blood-serum. The application of the results to the secretion of calcium in milk has been dealt with elsewhere [Wright, 1928].

Summary.

1. The unequal distribution of salts on either side of an artificial membrane in systems containing caseinogen, NaCl and $CaCl_2$, has been examined.

2. It is shown that the results may be quantitatively explained if two factors, the degree of dissociation of the protein salts and the establishment of a Donnan equilibrium, are taken into account.

3. The influence of one ion on the distribution ratios of other ions is demonstrated.

4. The applications of the results in the general study of the unequal distribution of salts in living tissues are briefly discussed.

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XLIV. THE ABSORPTION OF WATER BY GELATIN.

PART III. THE SULPHATE SYSTEM.

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In two previous papers [Jordan-Lloyd and Pleass, 1927, 1928] the absorption of water by gelatin in the chloride and nitrate systems has been described. The present paper deals with a system containing hydrogen, hydroxyl, sodium and sulphate ions and thus considers the specific effect of the bivalent sulphate ion. The water absorption has again been measured by the percentage change in weight, calculated on the air dry weight at 100°, of pieces of purified leaf gelatin when immersed in the experimental solutions, usually for a period of 3 days. The experimental material and method are the same as previously described with the exception of the important advantage of the use of the glass electrode for the determination of the p_H values. This has markedly decreased the magnitude of the errors occurring in the alkaline zone.

The influence of the three variables, p_H value, temperature (t) and concentration of sodium sulphate (M), on the water absorption of gelatin was investigated and the results are presented graphically.

It will be convenient again to consider separately the behaviour of the gelatin in the four zones of hydrogen ion concentration, centring respectively on p_H 2·6 (acid zone), p_H 10 (alkaline zone), p_H 5 (isoelectric zone), and p_H 7 (neutral zone).

When immersed in distilled water electrically neutral (isoelectric) gelatin absorbs water by imbibition. This water of imbibition may be regarded as penetrating through the system, forming a solid solution in the gelatin gel. It has been shown previously [Jordan-Lloyd and Pleass, 1928, p. 1013] that reduction of the water of imbibition leads to coagulation of the gel. In both acid and alkaline solutions, the gelatin, acting as a weak base or a weak acid, combines with the electrolyte present to form ionisable salts with a non-diffusible ion. This leads to the production of an internal osmotic pressure within the gel which induces further water absorption. This type of swelling is referred to as osmotic swelling. In solutions of salts at about p_H 5 gelatin absorbs more water than the water of imbibition. In these conditions the water absorption occurs by a different mechanism. Both positive and negative

ions of the salt are apparently absorbed equally by the gelatin, causing hydration of the gelatin. This type of swelling is referred to as lyotropic or salt swelling. In solutions of salts at about p_H 7 the absorption of water appears to be influenced both by osmotic swelling and lyotropic swelling.

The acid swelling zone (p_H 1–4).

The water absorption of gelatin at 18°, after immersion in solutions of sodium sulphate from 0 to 1·0 *M* concentration, plotted against the final p_H of the solution is shown in Fig. 1. When no sodium sulphate is present a curve for the swelling of gelatin in sulphuric acid solutions is obtained which is in many respects similar to the corresponding ones for hydrochloric and nitric acids, but which shows marked differences. Hydrochloric and nitric acids induce a maximum swelling of 6500 and 7000 % respectively on the dry weight of the gelatin at p_H 2·6, while the maximum in sulphuric acid is only about 5100 % and occurs at p_H 3·0.

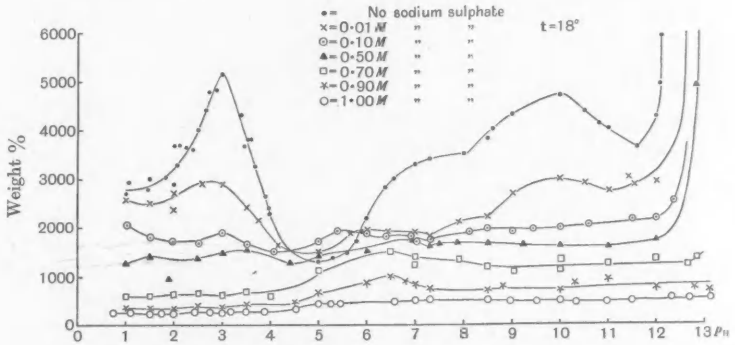

Fig. 1. p_H varying, *t* constant at 18°, *M* constant over a series of concentrations from zero to 1·0 *M*.

It is interesting to observe that from p_H 5·0 to 3·2 the curves for all three acids are similar, that is to say, in dilute solutions at any particular concentration up to 0·001 *N* the water absorption of gelatin will be the same in solutions of all three acids. At greater concentrations, however, the specific nature of the acids becomes apparent. The most arresting observation is that the p_H value of the position of maximum swelling is determined by the anion of the acid. Callow [1925], when measuring the rate of ice crystallisation in a gelatin gel, obtained p_H curves which he compares with Loeb's curves for the swelling of gelatin. The maxima of his ice crystallisation curves occur at p_H 2·5 in hydrochloric acid and at p_H 3·0 in sulphuric acid. Previously it has been stated [Loeb, 1922] that bivalent acids induce swelling which at the maximum is only half as great as that produced by univalent acids. This is not true in the conditions under investigation. The maximum swelling in

sulphuric acid is about 4/5 of the maximum swelling in hydrochloric or nitric acids. At p_H 2·5, however, the increase in swelling above that due to the water of imbibition is about twice as great in hydrochloric and nitric acids as it is in sulphuric, but it must be remembered that this is a comparison at one arbitrary p_H value and not a comparison of the magnitude of the swelling at the various maxima. Kuhn [1922] has measured the increase in volume of powdered commercial gelatin in solutions of hydrochloric, nitric and sulphuric acids. He finds that the maximum in solutions of nitric acid is slightly greater than the maximum in solutions of hydrochloric acid and considerably greater than the maximum in solutions of sulphuric acid. Also

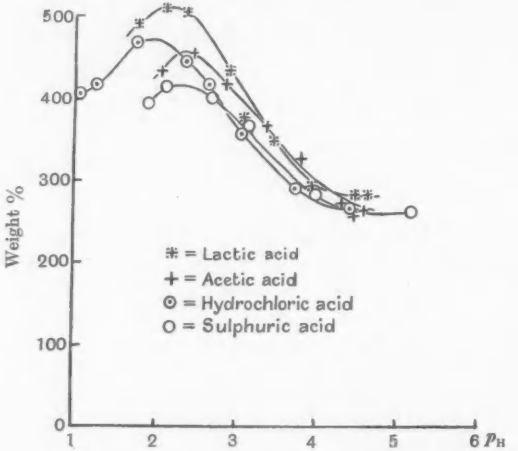

Fig. 2. The water absorption of collagen in lactic, acetic, hydrochloric and sulphuric acids at p_H values between 1 and 5.

Küntzel [1926] has measured the swelling of fibrous collagen by the contraction in length that occurs when the tendons from rats' tails are immersed in acid solutions. He states that nitric acid causes slightly greater maximum swelling than hydrochloric acid, sulphuric acid considerably less. The ratios of the magnitude of maximum swelling in sulphuric, hydrochloric and nitric acids are given in Table I. It is interesting to observe that results obtained by three very different methods indicate that although these monobasic acids induce greater swelling of proteins than sulphuric acid, the ratio of the

Table I. *The ratio of maximum swelling attained in sulphuric, hydrochloric and nitric acids.*

	H_2SO_4	HCl	HNO_3
Increase in weight of pure leaf gelatin (Jordan-Lloyd and Pleass)	1	1·27	1·37
Increase in volume of powdered commercial gelatin (Kuhn)	1	1·17	1·20
Contraction in the tendons of rats' tails (Küntzel)	1	1·26	1·33

maxima is, in every case, much less than 2. The results also confirm the fact that the swelling in nitric acid is only slightly, yet quite definitely, greater than that in hydrochloric acid. This difference in the degree of swelling is probably not due to the valency of the acid but to the nature of the anion.

Jordan-Lloyd and the author (unpublished results) have investigated the swelling of collagen in hydrochloric, sulphuric, lactic and acetic acids (Fig. 2). The experimental material was ox hide which had been limed to remove hair, albumins, globulins and mucoids and then, after neutralisation and washing free from mineral matter, had been dehydrated with acetone. Maximum swelling of collagen in the form of a fibrous tissue occurs in acid solutions at lower p_H values than that of gelatin in similar solutions. The degree of swelling is also much less [Jordan-Lloyd and Kaye, 1924]. The graphs show clearly that not only does the magnitude of the four maxima vary from 420 to 510 % on the dry weight, but that the p_H value at the maximum is specific for each acid. The maximum water absorption of collagen occurs at p_H 2·4 in sulphuric acid and at p_H 1·9 in hydrochloric acid solution. The curves indicate that in solutions of p_H values between 5·0 and 2·8 the water absorption in the presence of both hydrochloric and sulphuric acids is similar. In acetic and lactic acid solutions, however, the curves, even in this p_H range, are slightly removed from those for the inorganic acids, but as here the experimental error is much greater than in the gelatin systems this apparent difference may not be significant. Ostwald, Kuhn and Böhme [1925], when working with powdered gelatin in solutions of organic acids, obtained swelling curves which even for dilute solutions showed marked differences from one another. They state that swelling does not depend on the valency of the acid and that there is no correlation between the volume of maximum swelling in any acid and its ionisation constant.

Isgarischev and Pomeranzeva [1926] have measured the swelling of powdered caseinogen in $N/20$ solutions of 25 organic acids. No record is given of the p_H value of the solutions and the differences observed are certainly partially due to the variation in strengths of the acids. They state that there is no simple numerical relationship between the magnitude of the ionisation constant of the acid and the magnitude of the swelling of the caseinogen and suggest that the predominating factor is the nature and structure of the anion of the acid. In this connection probably one of the most important properties of the acid is its affinity for water. If the acid has a great affinity for water it is very probable that less water will be available for absorption by the gelatin. Fig. 2 shows that lactic acid, which has a tendency to form an anhydride at higher temperatures, *i.e.* an acid which has a negative affinity for water, induces a high degree of swelling, while sulphuric acid, which has a great affinity for water, especially at low p_H values, is, by some mechanism, preventing the gelatin from absorbing the water to so great an extent as in hydrochloric and nitric acid solutions. This view of the factors influencing swelling is supported by the influence of the presence of sodium sulphate,

which also in the anhydrous condition has a great affinity for water. The suppression of the acid swelling due to the presence of 0·01 *M* sodium sulphate is far greater than the corresponding suppression in the chloride and nitrate systems. When the concentration of the sulphate is increased to 0·5 *M* all acid swelling is completely suppressed and at greater concentrations of sodium sulphate the gelatin is coagulated to a high degree (see Figs. 1 and 9). This coagulation by sulphuric acid and sodium sulphate is much more intense and far more independent of the reaction of the solution than coagulation in either the chloride or nitrate systems. The pieces of gelatin on removal from *M* sodium sulphate solutions at all p_H values from 1–12 were very thin and brittle and rapidly dried in air, developing a surface efflorescence of sodium sulphate crystals. This suggests that the nature of the anion in both the pure acid solutions and in the salt solutions plays a very important part in determining the magnitude of the swelling. Also, in the sulphate system (see Fig. 9), when the water absorption at constant p_H values is plotted against the logarithm of the concentration of the sodium sulphate there is a point of inflexion on the curves at the point where the water absorption of the gelatin is equal to that in distilled water. This point corresponds to Loeb's theoretical deduction of the limiting value of the suppression of the swelling when the internal osmotic pressure is zero [Loeb, 1922].

The swelling of gelatin in all concentrations of sulphuric acid becomes greater as the temperature is increased (see Fig. 4). The influence of temperature on the water absorption of gelatin in acid solutions of varying concentrations of sodium sulphate at p_H 3·5 is shown in Fig. 3. It is of importance to note that the temperature coefficient of swelling becomes smaller as the salt concentration is increased. It may also be observed that, as in the chloride and nitrate systems, at all salt concentrations the temperature coefficient of water absorption is much greater above 12° than below this temperature; as this is true of the behaviour of the gelatin in the chloride nitrate and sulphate systems it strongly suggests that it is due to some inherent property of the gelatin and gives support to the theory of the transformation of the gel form (gelatin A) to the sol form (gelatin B), a change which begins at about 15° [Smith, 1919]. In sulphuric acid in the absence of inorganic salts and at reactions more acid than p_H 3·5 the gelatin dissolves at about 22°. Fig. 3 indicates that addition of increasingly large quantities of sodium sulphate not only decreases the swelling but also increases the temperature at which complete solution of the gelatin takes place.

The alkaline swelling zone (p_H 8–13).

At 18° the maximum swelling of gelatin in solutions of sodium hydroxide is 4700 % of its dry weight and occurs at p_H 10·0. As the alkalinity of the solution is decreased there is a rapid decrease in the water absorption until in solutions of p_H values between 8 and 7 the water absorption is nearly

constant and then in solutions of lower p_H value it more rapidly decreases again to reach the minimum at p_H 5.

In the alkaline zone the suppression of the swelling caused by the presence of sodium sulphate is much more pronounced than that caused by the presence of sodium chloride or sodium nitrate (see Fig. 1). Even a concentration of sodium sulphate as low as 0·01 *M* reduces the swelling at the alkaline maximum to 3000 % and 0·1 *M* completely masks the effect of the p_H value of the solution in the entire alkaline zone. In the two previous studies it was observed that sodium chloride reduced alkaline swelling, but at no concentration

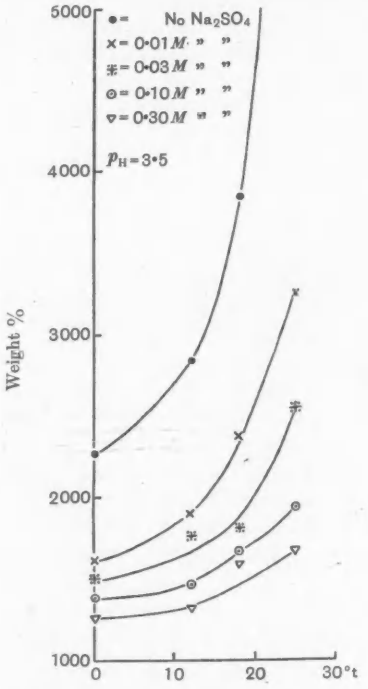

Fig. 3. p_H constant at 3·5, *t* varying from 0° to 25°, *M* constant at 5 values varying from 0 to 0·30 *M*.

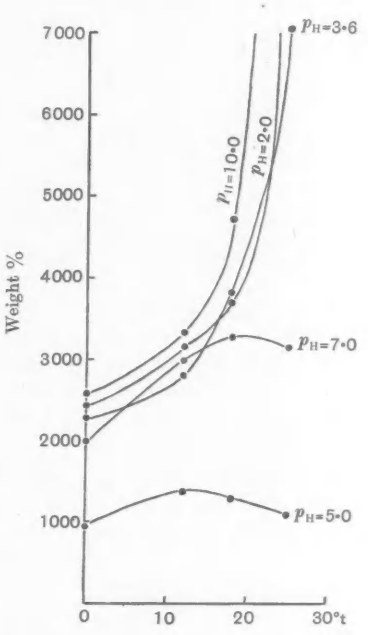

Fig. 4. *t* varying, *M* constant at zero, p_H constant at 2·0, 3·6, 5·0, 7·0 and 10·0.

up to 2 *M* was this salt able to suppress it completely, and that the influence of sodium nitrate was found to present a marked contrast. In solutions of concentrations of 0·1 *M* or less, the swelling was reduced although not entirely suppressed, but above this concentration sodium nitrate, by virtue of its solvent action, caused an increase in the water absorption until at concentrations of nitrate greater than 1·0 *M* the gelatin completely dissolved in solutions at all alkaline reactions. In the sulphate system, however, yet a

third type of salt behaviour is observed. Increasing the concentration of the sodium sulphate leads to a proportionate increase in the suppression of the swelling until at concentrations of sulphate greater than 0·7 *M* coagulation is induced and is intensified as the salt concentration is increased. In molar solutions of sodium sulphate the gelatin is highly coagulated, but not to quite such a high degree as in the corresponding acid solutions. The influence of the addition of sodium sulphate to solutions at p_H 10 on the water absorption of gelatin is shown in Fig. 9. Between values of 0·01 and 0·5 *M* the water absorption is inversely proportional to the logarithm of the salt concentration. At about 0·5 *M* there is a point of inflexion on the curve after which the swelling decreases much more rapidly and coagulation occurs.

The property of sodium sulphate of causing coagulation in moderately concentrated solutions is also accompanied by a reduction in the solvent action of the sodium hydroxide solutions. In pure sodium hydroxide solutions complete solution of the gelatin occurs at p_H 12·1, while in 0·1 *M* sulphate solutions the gelatin dissolves at p_H 12·6 and in 1·0 *M* solutions the gelatin has not dissolved even at p_H 13·1.

The isoelectric zone (p_H 4–6).

Minimum water absorption of gelatin occurs at p_H 5·0 if the solution is free from salts. The presence of sodium sulphate in fairly dilute solution causes an increase in the water absorption of gelatin in the isoelectric zone. As in the chloride and nitrate systems previously investigated, the salt swelling is proportional to the logarithm of the sodium sulphate concentration at concentrations between 0·01 and 0·1 *M* (see Fig. 9). In more concentrated solutions, however, the three systems exhibit marked differences from one another. In concentrated solutions of sodium chloride the logarithmic relationship continues to be mathematically exact up to 2 *M* concentration. Bechhold [1912] states that there is a maximum swelling induced in isoelectric sodium chloride solutions at 2·4 *M* concentration. In solutions of sodium nitrate more concentrated than 0·1 *M* lyotropic swelling increases more rapidly and is proportional to the nitrate concentration until in molar solutions the gelatin is on the verge of solution. In the sulphate system, however, it will be seen from Figs. 6 and 10 that as the concentration of sodium sulphate is increased from 0·1 to 0·5 *M* the swelling is practically constant, *i.e.* almost independent of the sulphate concentration, but at greater concentrations the swelling is inversely proportional to the salt concentration. Thus in the isoelectric range also, the magnitude of water absorption has been reduced to below that of the water of imbibition and coagulation is again encountered.

The influence of temperature on the water absorption of gelatin in solutions at p_H 5 is shown in Figs. 5 and 6. In the absence of salts, the water absorption increases gradually to a maximum as the temperature is increased from 0° to 12°. Above 12° the water absorption again decreases. In 0·01 *M*

sodium sulphate solution the water absorption increases regularly with temperature but as the sulphate concentration is increased to 0·1 *M* the temperature coefficient becomes increasingly larger at temperatures above 18°. In 0·3 *M* and 0·5 *M* solutions the temperature coefficient of water absorption is quite small. The curve of water absorption in molar sodium sulphate solution shows that less swelling occurs at higher temperatures. It should be noted, however, that the sodium sulphate is much less soluble at low temperatures and that a considerable quantity of the salt has crystallised out at 0°, leaving a solution which is less concentrated than molar.

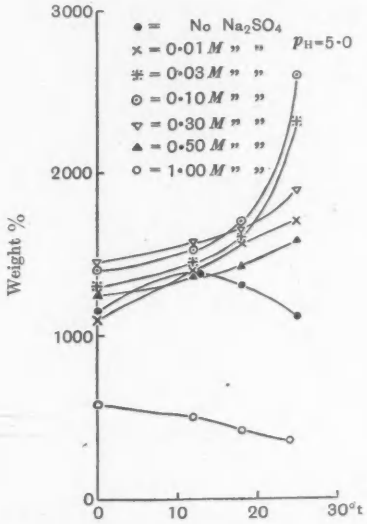

Fig. 5. *t* varying, p_H constant at 5·0, *M* constant at 7 values varying from 0 to 1·0 *M*.

At a temperature of 25° there is a clearly defined maximum of lyotropic swelling in 0·1 *M* sodium sulphate, greater concentrations strongly suppressing the swelling and causing coagulation of the gelatin (Fig. 6). At 0°, 12° and 18°, the maxima are not pronounced, the magnitude of the swelling at each temperature only varying slightly between 0·01 and 0·3 *M* sodium sulphate concentration.

The neutral zone (p_H 6–8).

It can be seen from Figs. 1 and 8 that at 18° the swelling of gelatin in dilute solutions of sodium hydroxide of p_H value between 6 and 8 is reduced by the addition of sodium sulphate in concentrations up to 0·02 *M*. Unlike its behaviour in similar solutions of the chloride or nitrate systems, the gelatin absorbs slightly more water as the sulphate concentration is increased

to 0·3 M after which further increase in the salt concentration leads to rapid decrease in the water absorption and coagulation. The influence of temperature on the water absorption at p_H 7·0 and 7·3 is illustrated by Figs. 7 and 8. In sodium sulphate solutions of concentration greater than 0·02 M, the curves in Figs. 6 and 8 show great similarity at temperatures of 0°, 12° and 18°, indicating that the magnitude of the swelling is similar in corresponding solutions at p_H 5 and 7. At 25°, however, the curve for swelling in solutions at p_H 7 does not exhibit a maximum at 0·1 M sodium sulphate concentration as does the curve for solutions at p_H 5. The water absorption of gelatin in

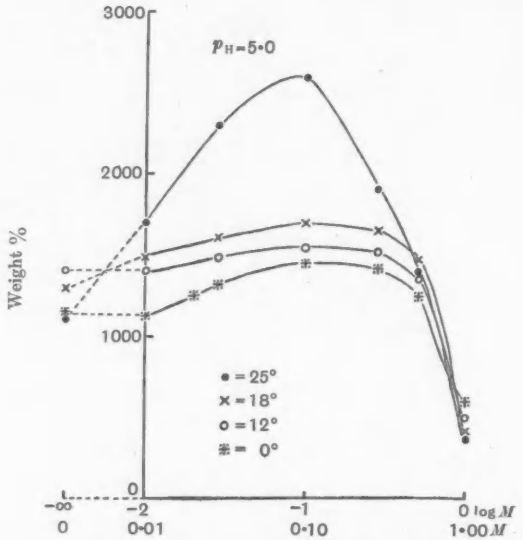

Fig. 6. M varying, p_H constant at 5·0, t constant at 0°, 12°, 18° and 25°.

solutions of varying concentration of sodium sulphate at p_H 7·3 plotted against the temperature is shown in Fig. 7. The maximum of water absorption, which occurs at about 15° in sodium hydroxide solutions of p_H 7·3, is absent when any sodium sulphate is present. The curves relating to all concentrations of sulphate from 0·01 to 0·3 M lie close together and show a gradually increasing temperature coefficient as the temperature is increased. In molar solutions increase in temperature causes decrease in water absorption by the gelatin. This increase in water absorption of gelatin at low temperatures may be affected to some extent by the decreased solubility of the sodium sulphate. In moderately concentrated solutions the sodium sulphate produces the predominating effect.

It can be seen in Fig. 10 that at all p_H values and at all concentrations

of sodium sulphate solutions from 0·1 to 1·0 *M* the magnitude of the swelling is determined by the sulphate concentration almost independently of the p_H value of the solution.

Discussion.

The behaviour of gelatin in the presence of dilute solutions of sulphuric acid and sodium sulphate is to some extent similar to its behaviour in corresponding solutions in the chloride and nitrate systems, but, even in dilute

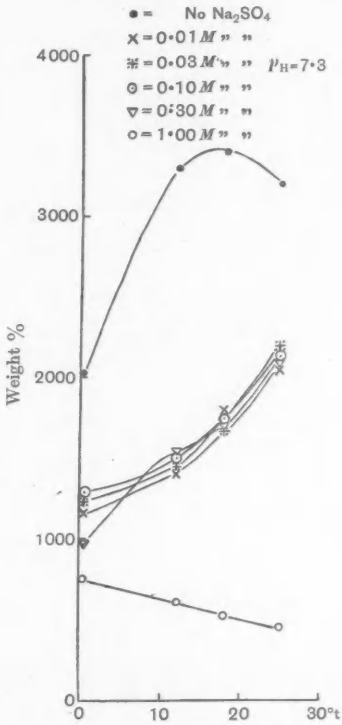

Fig. 7. *t* varying, p_H constant at 7·3, *M* constant at 5 values varying from zero to 1·0 *M*

solutions and to a much greater extent in concentrated ones, marked differences are patent.

From a physico-chemical view-point the three acids and their corresponding sodium salts are similar in many respects but the sulphate ion is differentiated by its bivalent character. Sulphuric acid also is not ionised to so great an extent and is only about half as strong as nitric and hydrochloric acids and has a great affinity for water.

It should be borne in mind when comparing the effect of sodium sulphate

with that of sodium chloride and nitrate that the unit of concentration used is molecular and that equal molecular concentrations do not indicate equal ionic concentrations. This is particularly important when considering osmotic effects. The three systems which have been investigated form a very convenient triad. The hydrochloric acid, sodium chloride, sodium hydroxide system presents what may be regarded as the simplest type, *i.e.* the salt action is almost entirely confined to suppression of osmotic swelling in the acid and alkaline zones and to inducing lyotropic swelling near the isoelectric point.

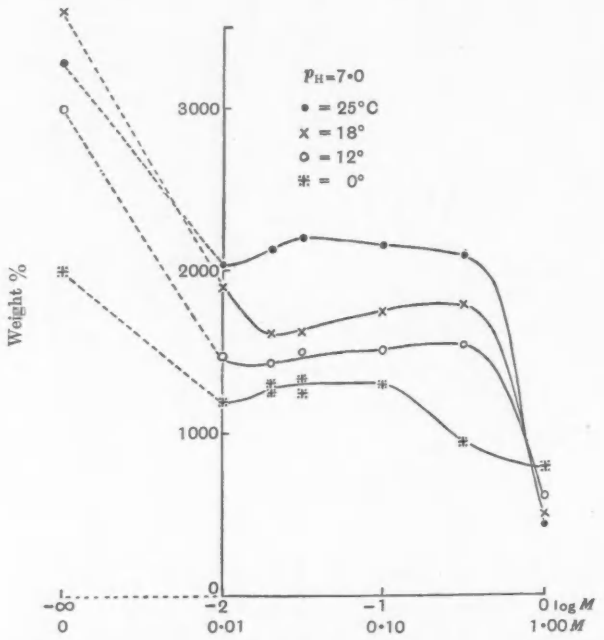

Fig. 8. M varying, p_H constant at 7·0, t constant at 0°, 12°, 18° and 25°.

The behaviour of gelatin in the nitrate system is similar in dilute solutions to that in the chloride system but, as the concentration of the nitrate is increased, proportionately greater swelling is induced which becomes more and more predominant and leads to solution of the gelatin. In solutions of nitric acid the behaviour of gelatin is similar to that in hydrochloric acid, but the magnitude of the water absorption is slightly greater. The suppression of swelling by sodium nitrate in acid solutions and the induction of lyotropic swelling in solutions of 0 to 0·7 M concentration at p_H 5 are slightly greater than the corresponding effects in the chloride system. As the concentration of sodium nitrate is increased, however, this greater lyotropic swelling proves

to be the precursor of solution, which takes place in 2 M solutions at all p_H values greater than 2·5 and in molar solutions at all p_H values above 5. In the nitrate system coagulation of the gelatin may occur to a greater degree than in the chloride system but its occurrence is far more restricted by the

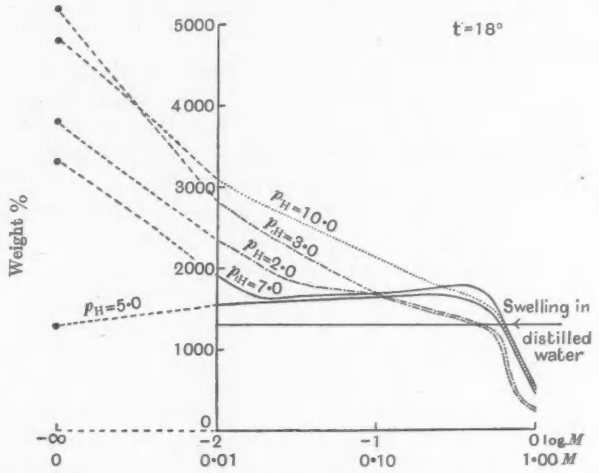

Fig. 9. M varying, t constant at 18°, p_H constant at 2·0, 3·0, 5·0, 7·0 and 10·0

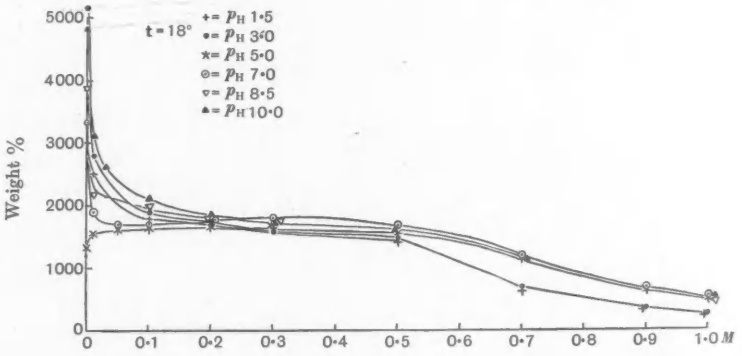

Fig. 10. M varying, t constant at 18°, p_H constant at 1·5, 3·0, 5·0, 7·0, 8·5 and 10·0.

reaction of the solution, due to the fact that the concentrated nitrate solutions exhibit a powerful solvent action. The powerful coagulating action which occurs in sulphate solutions presents a marked contrast. In sulphuric acid solutions of p_H values of 3·0 or less, the magnitude of the swelling of gelatin

is considerably less than in the nitrate and chloride systems. As the concentration of the sodium sulphate solutions at p_H 5 is increased, the lyotropic swelling of gelatin also is suppressed by the coagulating action of the concentrated solutions and in acid solutions the suppression of the osmotic swelling is enhanced. Thus, as the sulphate concentration is increased, coagulation, which is antagonistic to both osmotic and lyotropic swelling, becomes the predominant feature at all reactions.

It has previously been suggested [Jordan-Lloyd and Pleass, 1928] that if coagulation is defined as the reduction of the concentration of water in the gel to below that characteristic of the water of imbibition of the gelatin then the degree of coagulation may be expressed numerically. If the "coagulation ratio" be defined as the ratio of the percentage weight of the gelatin in distilled water to its percentage weight in the conditions under consideration, we may compare the degree of coagulation in different systems. It should be noted that a coagulation ratio of 1 indicates that there is neither swelling nor coagulation. Numbers greater than unity indicate coagulation and less than unity, swelling. In distilled water the swelling of gelatin is 1300 %, while in molar sodium sulphate solution at p_H values from 1–4 it is 250 %. Therefore in these conditions the degree of coagulation is 1300/250 = 5·2. Similarly, at molar concentrations at p_H 2 in the chloride and nitrate systems the coagulation ratio is 1·8 and 2·2 respectively. At p_H 2 in 2 *M* chloride and nitrate solutions the coagulation is 4·0 and 3·6 units respectively. In molar sodium sulphate solution the coagulation ratio slightly decreases as the reaction becomes more alkaline. At p_H 5 the value is 2·9 units, while at p_H values greater than 7 the coagulation is 2·6 units.

The coagulation in rather more dilute solutions of sodium sulphate is very interesting.

	Coagulation ratio	
p_H	0·9 *M*	0·7 *M* sodium sulphate
2·0	2·6	2·0
4·0	2·9	1·9
5·0	2·0	1·2
6·5	1·3	0·8
7·0	1·6	0·9
9·0	1·7	1·1
12·0	1·5	1·0

At p_H 6·5 both the curves for 0·9 and 0·7 *M* sodium sulphate show a minimum of coagulation.

The curves in Fig. 10 which indicate the influence of salt concentration at various reactions clearly show that although at salt concentrations from 0·1 to 0·5 *M* the p_H value of the solution has very little effect on the water absorption, at greater concentrations of sodium sulphate, which lead to coagulation of the gelatin, the degree of coagulation is slightly greater in acid than in neutral or alkaline solutions.

It is interesting to observe that coagulation of the gelatin increases its resistance to solution; this is true both of solution caused by increasing the

temperature and solution induced in alkaline fluids of high p_H value. This suggests that coagulation is the converse of lyotropic swelling in this respect also, for swelling appears to precede and promote solution. Thus it appears that the relative concentration of water and gelatin of the aqueous phase of the gel greatly influences the solubility of the gel in water. This suggestion is supported by the fact that dry isoelectric gelatin has a very low solubility in water, even at 100°, whereas if the same sample of gelatin be soaked in water at room temperature for 24 hours, the moist gelatin may then be melted at temperatures above 37° with the formation of quite concentrated solutions (15 % or even greater).

The investigation of the water absorption of gelatin in the sulphate system suggests that in very dilute solutions the action of sulphuric acid and sodium sulphate is very similar to the action of corresponding solutions in the chloride and nitrate systems but as the concentrations are increased the specific nature of the ions becomes more and more the dominating effect. In isoelectric solutions of sodium chloride, nitrate and sulphate as the concentrations of the salts are increased the divergence between the magnitudes of the lyotropic swelling becomes gradually more pronounced. The form of the curves, shown in this and the two previous papers indicating a high degree of swelling leading to solution of the gelatin in the case of the nitrate and coagulation of the gelatin in the case of the sulphate solutions, suggests that with increasing concentration of salt the specific influence of the anions finally overwhelms the influence of the hydrogen and of the hydroxyl ions at all values of p_H.

Summary.

1. Maximum swelling of gelatin occurs in a solution of sulphuric acid at p_H 3·0.

2. Sodium sulphate in concentrations up to 0·5 *M* in the presence of sulphuric acid suppresses the osmotic swelling of the gelatin due to the acid. At greater concentrations of this salt there is coagulation of the gelatin.

3. Increasing the temperature of solutions in the acid zone causes an increased water absorption by the gelatin. The temperature coefficient of swelling is greater the higher the temperature, but is decreased by an increase in the concentration of sodium sulphate.

4. The osmotic swelling of gelatin in alkaline solutions is suppressed to a greater degree by solutions of sodium sulphate than by equal molar concentrations of sodium chloride or nitrate. At concentrations of sodium sulphate of 0·7 *M* or greater, coagulation is induced.

5. At the isoelectric point of gelatin the water absorption in solutions of sodium sulphate is proportional to the logarithm of the salt concentration up to 0·1 *M*. At greater concentrations lyotropic swelling is reduced and coagulation occurs in solutions of greater than 0·7 *M* concentration.

6. In solutions of sodium sulphate of 0·1 to 1·0 *M* concentration the magnitude of the water absorption of the gelatin is determined chiefly by the sulphate concentration almost independently of the p_H value of the solution.

7. A method of interpreting the term "coagulation" on a numerical scale is described.

8. Gelatin in the coagulated condition tends to resist the solvent action of alkaline solutions.

9. Increase in temperature generally causes greater water absorption, the temperature coefficient becoming larger as the temperature is increased.

In conclusion, the author wishes to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper and to express her gratitude to the Director, Dr Jordan-Lloyd, for her advice and helpful criticism during the progress of this work.

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XLV. OBSERVATIONS ON THE IODINE-CONTAINING COMPOUNDS OF THE THYROID GLAND. ISOLATION OF *dl*-3:5-DIIODOTYROSINE.

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About three years ago one of the authors [Harington, 1926] described an improved method for the isolation of thyroxine from the thyroid gland, which was based on a graduated hydrolysis of the gland substance with barium hydroxide. When desiccated thyroid was boiled with dilute barium hydroxide the iodine was separated into three fractions, the first being contained in the precipitate of insoluble barium salts, the second being obtainable by acidification of the alkaline filtrate, and the third remaining soluble in presence of acid. Thyroxine was isolated by subjecting the first two fractions to more intensive hydrolysis with barium hydroxide, whilst no evidence could be obtained of the presence of thyroxine in the third, acid-soluble, portion. A similar separation of the iodine compounds of the thyroid into acid-soluble and acid-insoluble had been effected previously by Kendall by hydrolysis with dilute sodium hydroxide, and he was able to show [1919] that the physiological activity was confined to the acid-insoluble fraction, *i.e.* to that fraction from which alone thyroxine could be isolated. This observation has been confirmed by an experiment kindly performed for us by Dr J. H. Gaddum. Desiccated thyroid gland was boiled with 10 % crystalline barium hydroxide under the usual conditions and the hydrolytic products were separated into acid-insoluble and acid-soluble fractions; injection of 2·6 mg. per kg. of the iodine of the acid-insoluble portion produced an increase of 60 % in the oxygen consumption of a rat, whilst a definitely smaller effect was obtained after injection of 26 mg. per kg. of the iodine of the acid-soluble fraction. It is evident, therefore, that this preliminary hydrolysis, although it is of so mild a character that it cannot conceivably involve any destruction of thyroxine, effects a practically complete separation of the latter from the other iodine-containing compounds which may be present.

At the time of the previous investigation the isolation of thyroxine itself was the exclusive object in view and the further examination of the acid-soluble fraction was therefore postponed. The present communication deals

with the results of this examination, leading eventually to the isolation of 3 : 5-diiodotyrosine, and we shall adduce evidence in support of the view that the whole of the acid-soluble iodine is indeed present as diiodotyrosine, whilst the whole of the acid-insoluble iodine belongs in all probability to thyroxine.

If the alkaline filtrate obtained after hydrolysis of desiccated thyroid gland with 10 % barium hydroxide be carefully acidified with hydrochloric acid (to about p_H 5·0) the thyroxine-iodine is, as previously indicated, almost quantitatively precipitated; after removal of this precipitate one is left with a solution containing 50 % or more of the original iodine of the gland, practically the whole of which remains in organic combination. The solution so obtained is, however, of little use for further investigation, owing to its content of barium chloride. Our first step was, therefore, to substitute sulphuric acid for the hydrochloric acid formerly used for the precipitation of the thyroxine fraction. This step resulted in the removal of a certain proportion of the acid-soluble iodine by adsorption on the precipitate of barium sulphate and thyroxine; this amount could be easily recovered, however, by extraction of the precipitate with alkali, removal of the barium sulphate, and acidification, when the thyroxine was again precipitated, and the filtrate containing the acid-soluble iodine which had been removed by adsorption on the barium sulphate could be reunited at a later stage with the main acid-soluble fraction.

To return to the latter, in the first experiment, the solution, which gave a strong biuret reaction and evidently contained for the most part peptones and other higher protein degradation products, was concentrated and subjected to more intensive hydrolysis either with acid or alkali; hydrolysis with acid was soon dismissed as useless, since it resulted in the entire destruction of the organic iodine compounds, the iodine appearing in the solution of hydrolytic products as iodide. Hydrolysis with barium hydroxide was evidently much more favourable since a large proportion of the iodine remained in organic combination; no success, however, attended attempts to isolate the iodine compound from the hydrolytic products. Nevertheless these early experiments were useful in that they gave an indication of the probable nature of the compound for which we were searching; for it was observed that, after partial separation of the hydrolytic products, those fractions which were rich in iodine gave the colour reaction with nitrous acid and ammonia which is given also by thyroxine, and which was previously shown [Harington and Barger, 1927] to be characteristic of the *o*-diiodophenolic grouping, and was put on a roughly quantitative basis by Ingvaldsen and Cameron [1926]. At this stage we returned to the original solution to see whether a preliminary partial separation of the iodine compound might not be effected at this early stage, and the mixture of products obtained after the second hydrolysis be thereby simplified. We found that precipitation with basic lead acetate served our purpose in this respect. Addition of basic lead acetate to the neutralised solution until no further immediate precipitate was formed removed about 80 % of the iodine; the precipitation of the iodine could indeed be made

quantitative by making the solution alkaline with ammonia, but the last 20 % of the iodine was accompanied by so large a preponderance of other material that we found ourselves finally in no better position than before the lead treatment; we therefore contented ourselves with the 80 % precipitation obtained as indicated above. The lead salts were filtered off and decomposed with sulphuric acid, and the filtrate, after removal of sulphuric acid, was united with the portion recovered, as described above, from the first acid precipitate, and was concentrated and further hydrolysed with 40 % barium hydroxide. After removal of barium with carbon dioxide the solution was treated with silver nitrate which precipitated the whole of the iodine; the silver salts were extracted with dilute nitric acid, the iodide formed during the hydrolysis being left undissolved, and were reprecipitated with ammonia; they were then decomposed with hydrogen sulphide in the usual way. We thus obtained a solution which gave a strong nitrous acid reaction, and it became more and more evident that the properties of the substance under investigation were closely similar to those of diiodotyrosine.

Earlier workers who have isolated this compound from natural sources [cf. *e.g.* Wheeler and Mendel, 1910] have employed hydrolysis with barium hydroxide followed by silver precipitation, and have then utilised phosphotungstic acid in order to separate the diiodotyrosine from the dicarboxylic amino-acids which are the chief accompanying impurity. In our hands this reagent did not prove satisfactory either from the point of view of completeness of precipitation of the iodine, or from that of cleanness of separation. We therefore had recourse to extraction of the neutralised solution with butyl alcohol for the purpose in view. Continuous extraction with this solvent is undesirable, since, on prolonged boiling with butyl alcohol, diiodotyrosine appears to undergo some decomposition; by shaking out the warm solution with successive quantities of butyl alcohol [cf. Onslow, 1921] we were, however, successful in extracting the greater part of the iodine with no disadvantageous effects. The aqueous solution of the material extracted by butyl alcohol still contained some colloidal substances which inhibited the crystallisation of the diiodotyrosine; these substances could be removed by treatment, under the appropriate conditions, with uranium acetate, and then, after one more precipitation as the lead salt, the diiodotyrosine could be obtained crystalline without difficulty.

Reference to the experimental part below, and to the diagrammatic representation of a typical experiment, will show that, although the actual quantity of diiodotyrosine isolated amounts only to about 11 % of the total iodine of the thyroid, the losses during the process of isolation are essentially the continuous small losses of material which are inevitably associated with a somewhat complex manipulation of this type. An exception to this statement may at first sight appear to be indicated by the large loss of iodine as iodide during the intensive hydrolysis with barium hydroxide; we have satisfied ourselves, however, that even pure diiodotyrosine admixed with a protein

and subjected to similar treatment, loses a considerable proportion of its iodine as iodide, and such loss may well be greater when the compound is at the same time being split off from peptide combination; we do not, therefore, regard this phenomenon as invalidating our general argument that at no stage is there any sharp break in the recovery of the iodine, such as would indicate the presence of an iodine-containing compound of a different character. In order further to confirm this conclusion we carried out, in one experiment, colorimetric determinations by the nitrous acid reaction coincidently with our organic iodine determinations, and, although no pretence is made that the colorimetric results were more than approximations, a definite parallelism between the two sets of observations was apparent[1].

Having then convinced ourselves that we could account for all the acid-soluble iodine as diiodotyrosine, we returned to the acid-insoluble fraction, to see whether we could find here any indication of the presence of an organic iodine-containing compound other than thyroxine. In the method previously described [Harington, 1926] the thyroxine was recovered by extraction with alkaline sodium sulphate; this manipulation is troublesome, and it has been found better to decompose the salts by suspension in warm dilute hydrochloric acid; lipoidal material is then removed with ether, and the remaining insoluble precipitate is united with the main acid precipitate for the second hydrolysis. It was formerly stated that, at the end of the second hydrolysis, the whole of the thyroxine was to be found in the precipitate of barium salts; this remark requires modification. The distribution of thyroxine at this stage depends on the concentration of iodine in the solution; if this concentration (at the commencement of the hydrolysis) is, as in the earlier experiments, 1 mg. per cc. or more, it is true that most of the thyroxine appears in the precipitate, although even here traces may be obtained by acidification of the mother liquor; on the other hand, with more dilute solutions, a large part of the thyroxine may remain in solution; in any case, in carrying out this isolation, the solution should at this stage be acidified and any precipitate which is obtained should be combined with the material obtained on recovery from the insoluble barium salts; the combined acid-insoluble material is then converted into crystalline thyroxine by the method previously described [Harington, 1926].

Reference to the experimental part will show that here again we meet, in the process of isolation of thyroxine, with a series of losses such as can

[1] The quantitative nitrous acid reaction in coloured solutions is conveniently carried out as follows. Into each of two test tubes A and B is introduced a 5 cc. sample of the solution to be tested containing approximately 0·5 mg. of iodine per cc. To A is added 1 cc. of a 0·1 % solution of diiodotyrosine, to B 1 cc. of water. To both tubes are added two drops of 30 % sodium nitrite and 3 drops of concentrated hydrochloric acid; the tubes are shaken and allowed to stand 2 minutes at the ordinary temperature; to each is added 9 cc. of butyl alcohol, the contents being shaken for 1 minute and allowed to separate; 5 cc. of each butyl alcohol layer are transferred to the cups of a colorimeter and 0·5 cc. alcoholic ammonia (0·5 *N*) added; the resulting pink colours are compared immediately.

reasonably be ascribed to unavoidable imperfections of technique. In the particular experiment described, the thyroxine ultimately isolated represented 16 % of the total iodine of the gland substance; this is an average result with the improved technique now employed; we have had even better yields. As in the case of diiodotyrosine, so with thyroxine, the greatest loss occurs at the stage of the intensive alkaline hydrolysis. This loss consists in part of iodine split off as iodide, and for the rest of iodine which is still in organic combination but is soluble in acid. The proportion which appears in the inorganic condition is considerably less than is the case at the corresponding stage in the isolation of diiodotyrosine; this difference, we think, is due to the fact that a great part of the thyroxine, as it is set free by hydrolysis, separates as the sparingly soluble barium salt and is thus removed from the action of the alkali, whilst the soluble diiodotyrosine remains exposed to this action throughout the experiment. As to the further small amount of iodine which appeared at this stage in the acid-soluble organic form (an amount so small as to preclude its detailed examination) two obvious possibilities suggest themselves; in the first place it is naturally not claimed that the division of the products of the preliminary hydrolysis of the thyroid into those containing thyroxine on the one hand and those containing diiodotyrosine on the other is absolutely quantitative; indeed the experiment of Dr Gaddum mentioned above shows that this is not the case, since a trace of residual physiological activity was found in the acid-soluble fraction; if, then, traces of thyroxine may appear among the acid-soluble products, it is equally possible that traces of diiodotyrosine may appear among the acid-insoluble substance; especially does this seem likely to us since one of our principal difficulties in the isolation of diiodotyrosine has been the extreme ease with which, from neutral or slightly acid solutions, this substance is adsorbed on precipitates; it is not unreasonable, therefore, to suppose that part of the fraction of the iodine under consideration belongs in fact to diiodotyrosine. A second possibility which must not be ignored is that, under the somewhat severe conditions of the hydrolysis, a part of the thyroxine may yield a product still containing organically-combined iodine but soluble in acid. Thus it is only those two iodine atoms which are situated *ortho* to the phenolic group of thyroxine which we should expect to be labile towards alkali of the concentration employed during the hydrolysis; were hydrolysis of these two iodine atoms to occur we should have a pyrogallol derivative still containing iodine, which would almost certainly be fairly soluble in water, and such part of which as was not further oxidised would go to make up the fraction which we are discussing. In any case we find it more reasonable to suppose that this small fraction of acid-soluble organic iodine (which is, after all, only 5 % of the total iodine) appears at this stage for some such reason as we have suggested, rather than that it indicates the presence of an independent iodine-containing compound in the original gland.

On the basis of this investigation, therefore, we feel justified in advancing

the definite view that there are only two iodine-containing compounds in the thyroid gland, namely thyroxine and 3 : 5-diiodotyrosine; in the gland which we have used for our experiments, the iodine would appear to be about equally distributed between these two compounds. This gland material, which we have employed throughout, is all obtained from one geographical source, and it has, moreover, shown a remarkable constancy of iodine content at whatever season of the year it has been purchased. There is, however, little doubt that variations in the distribution of iodine in the thyroid between diiodotyrosine and thyroxine may occur. Such variations would account for the lack of parallelism between iodine content and physiological activity which has been observed in the past for different samples of thyroid, and for the observations of Kendall and Simonsen [1928] and others on the varying proportion of the iodine which can be obtained in the acid-insoluble condition; it has indeed been stated that in some glands the iodine is entirely in the acid-soluble condition. The suggestion has already been advanced [Harington and Barger, 1927] that, biologically, thyroxine is derived from tyrosine through the stage of 3 : 5-diiodotyrosine, two molecules of which may be supposed to undergo oxidative coupling with the loss of one side-chain to give thyroxine; the actual isolation of diiodotyrosine from the thyroid lends strong support to this theory. If we are to regard diiodotyrosine as the precursor of thyroxine, it is evident, that with varying states of activity of the gland we must expect to find varying relationships between the amount of the precursor and of the complete hormone. Taking the possibility of such variations into account, an obvious corollary is that the only reasonable chemical assay of the therapeutic value of a thyroid preparation must be based on the acid-insoluble iodine and not on the total iodine content of the material.

Brief reference must here be made to a publication by Kendall and Simonsen [1928] which has recently appeared. These authors cite their own observations and those of other workers to the effect that the whole of the physiological activity of the thyroid gland cannot be accounted for by its thyroxine. The discrepancy to which Kendall draws attention is, however, not so much between the activity of a gland and its content of thyroxine, as between the activity and the amount of thyroxine which can be isolated. We think that most workers who have had experience of this type of manipulation will agree that a very grave difference exists between the amount of a compound which is present in a tissue and the amount of that compound which can be isolated in the pure condition. We have attempted above to bring forward evidence that the whole of the acid-insoluble iodine of the thyroid may be regarded as thyroxine; if this conclusion can be accepted and if we bear in mind that the acid-insoluble fraction usually represents 40 to 60 % of the total iodine, and further, that the true physiological activity of thyroxine as it occurs in the thyroid may well be different from that of the free compound, the discrepancy no longer appears so serious. It may be recalled that the physiological activity of laevorotatory thyroxine was found to be

definitely greater than that of the dextrorotatory isomeride, whence it was assumed that the former was probably the naturally occurring compound [Harington, 1928]. Recent experiments in this laboratory have cast some doubt on the correctness of this deduction, and, until the point is finally cleared up, we do not wish to base any arguments on the optical activity of the naturally occurring hormone. There remains, however, the possibility that the physiological activity of thyroxine in the natural state, *i.e.* in peptide or other form of combination, may be enhanced with respect to its activity in the free condition. We suggest that, in the present state of our knowledge, any remaining discrepancy between the physiological activity and the acid-insoluble iodine content of the thyroid may be explained on these lines more acceptably than by the gratuitous assumption of the existence in the gland of an "active" form of thyroxine differing in chemical structure from the compound as we know it.

Experimental.

In this part a detailed description is given of one complete experiment which is typical of several; for the sake of clarity a diagrammatic representation of the process is given.

Desiccated thyroid gland (250 g. containing 1·220 g. of iodine) was boiled under a reflux condenser for 6 hours with 2500 cc. of a 10 % solution of crystalline barium hydroxide; after cooling, the solution was filtered, and the filtrate brought to p_H 5·0 by addition of 50 % sulphuric acid; the precipitate of thyroxine and barium sulphate, which carried down also some of the acid-soluble iodine by adsorption, was filtered off, and the filtrate and washings (A) containing 515 mg. of iodine were set aside.

(1) *Isolation of thyroxine.* The precipitate of insoluble barium salts resulting from the preliminary hydrolysis was ground up and suspended in dilute hydrochloric acid; the solution was brought to the boil, adjusted to p_H 5·0 and filtered; the precipitate was ground up with ether, again filtered, and dissolved in dilute sodium hydroxide; the resulting solution was combined with that resulting from extraction of the barium sulphate-thyroxine precipitate with warm dilute sodium hydroxide followed by removal of the barium sulphate; the combined sodium hydroxide solutions contained 549 mg. of iodine. The total loss up to this stage was therefore 156 mg. of iodine; this was accounted for in part by a small unhydrolysed residue of the original material, and, for the rest, was spread over the various operations above described; in numerous experiments the distribution of these losses, as between the different operations, was uniform, and each individual loss was small. The sodium hydroxide solutions were acidified with sulphuric acid to p_H 5·0 and the precipitate was filtered off; the solution was treated with slight excess of barium hydroxide and the filtrate (B) containing 118 mg. of iodine was reserved (see below). The acid-insoluble precipitate (431 mg. of iodine) was dissolved in 300 cc. of water with the aid of a little ammonia,

Table I. *Separation of iodine into acid-soluble and acid-insoluble fractions.*

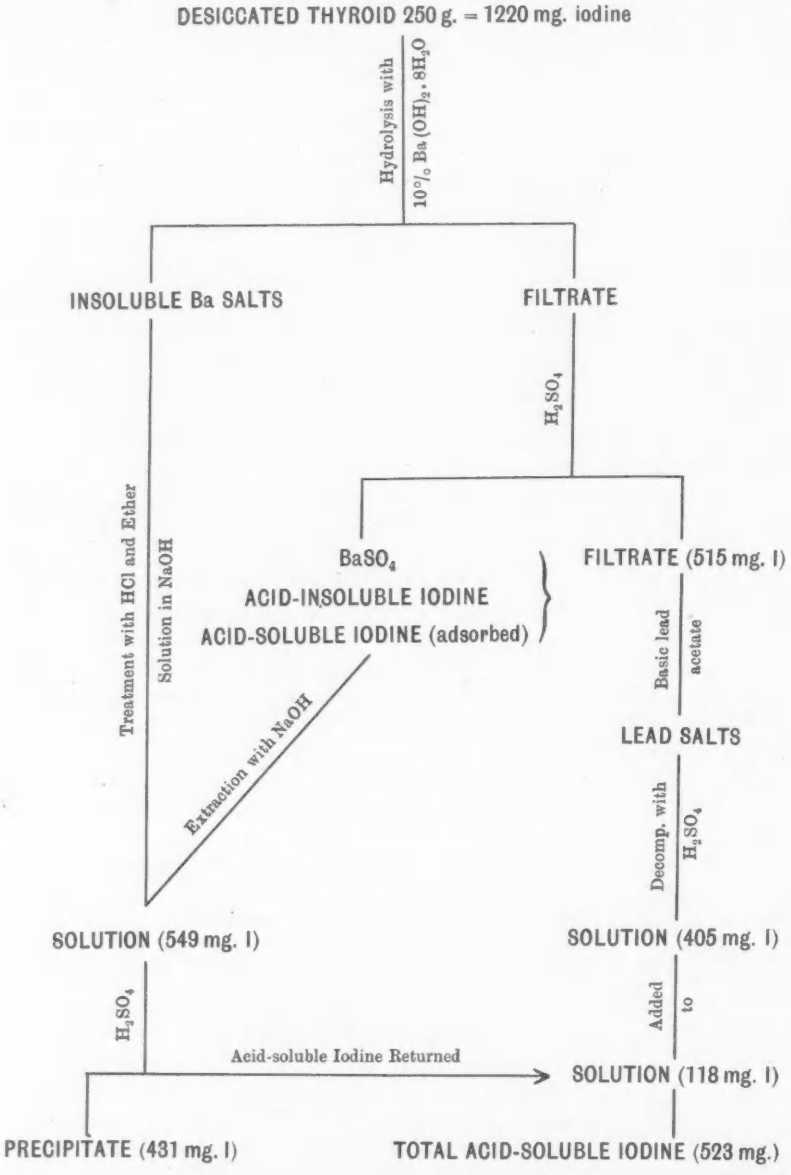
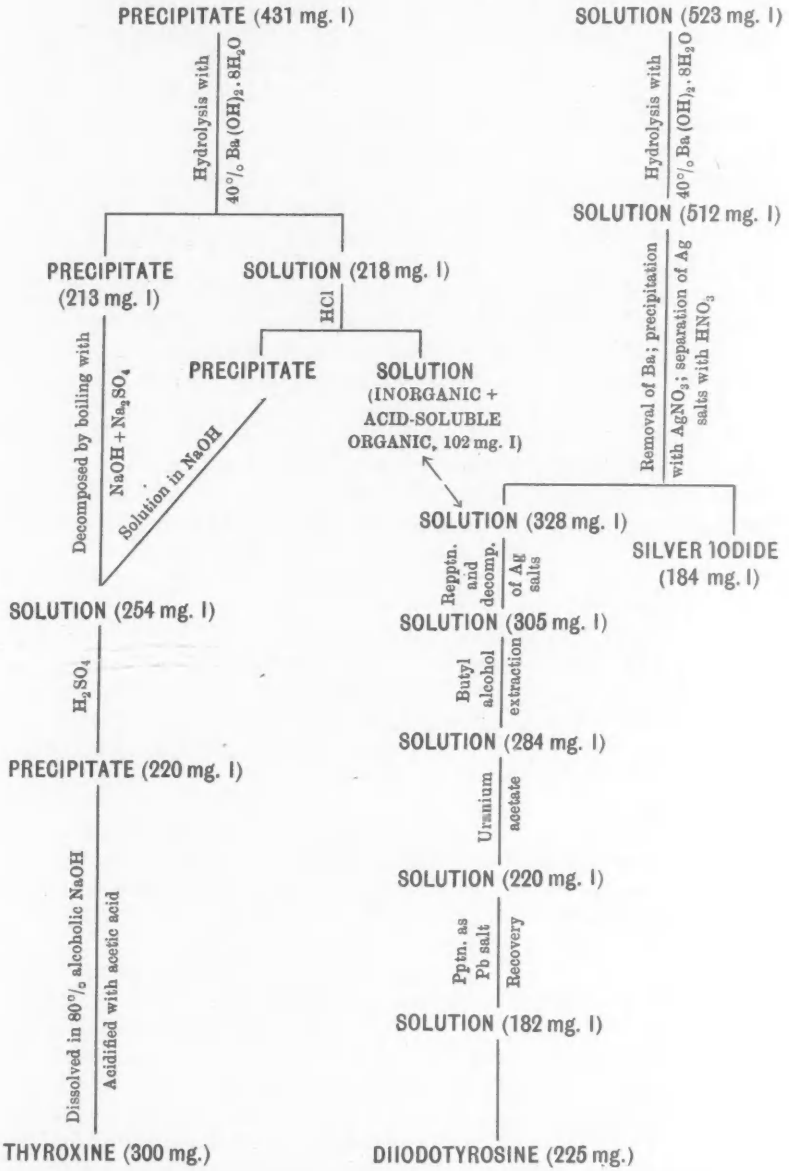


Table II. *Isolation of thyroxine and of diiodotyrosine.*


PRECIPITATE (431 mg. I)
Hydrolysis with 40% $Ba(OH)_2.8H_2O$
SOLUTION (523 mg. I)
Hydrolysis with 40% $Ba(OH)_2.8H_2O$
SOLUTION (512 mg. I)
Removal of Ba; precipitation with $AgNO_3$; separation of Ag salts with HNO_3
PRECIPITATE (213 mg. I)
SOLUTION (218 mg. I)
HCl
Decomposed by boiling with $NaOH + Na_2SO_4$
PRECIPITATE
Solution in NaOH
SOLUTION (INORGANIC + ACID-SOLUBLE ORGANIC, 102 mg. I)
SOLUTION (328 mg. I)
SILVER IODIDE (184 mg. I)
Repptn. and decomp. of Ag salts
SOLUTION (254 mg. I)
SOLUTION (305 mg. I)
H_2SO_4
Butyl alcohol extraction
PRECIPITATE (220 mg. I)
SOLUTION (284 mg. I)
Uranium acetate
SOLUTION (220 mg. I)
Pptn. as Pb salt
Recovery
SOLUTION (182 mg. I)
Dissolved in 80% alcoholic NaOH
Acidified with acetic acid
THYROXINE (300 mg.)
DIIODOTYROSINE (225 mg.)

crystalline barium hydroxide was added to 40 % concentration, and the whole was heated for 18 hours at 100°; the solution was filtered hot, the filtrate cooled and the barium hydroxide which separated was removed; hydrochloric acid was then added to p_H 5·0 and the precipitate (C) collected; the hydrochloric acid filtrate contained 102 mg. of iodine (in a parallel experiment this was shown to consist to an almost equal extent of inorganic iodide and acid-soluble organic iodine). The insoluble barium salts were decomposed by boiling with alkaline sodium sulphate, and to the alkaline filtrate was added the hydrochloric acid precipitate (C); the alkaline solution was heated to boiling and brought to p_H 5·0 with sulphuric acid; the precipitate was collected and dissolved in 80 % alcohol with the aid of sodium hydroxide; the solution was filtered, heated to boiling, and acidified with acetic acid, when there separated 300 mg. of thyroxine. The serious loss in this process occurs therefore during the drastic hydrolysis; the remaining loss is accounted for by the additive effect of incomplete recovery from the insoluble barium salts, incomplete re-precipitation, and finally incomplete separation on crystallisation from acetic acid-alcohol, each individual loss again being small in amount.

(2) *Isolation of dl-3 : 5-diiodotyrosine.* The filtrate A (see above) was treated with basic lead acetate (the B.P. solution) until no further immediate precipitation occurred; after standing overnight the lead salts were filtered off and suspended in water (2000 cc.); the mixture was heated to boiling and 50 % sulphuric acid was added until the reaction remained acid to Congo red; lead sulphate was removed by filtration and the filtrate was freed from sulphuric acid by addition of a slight excess of barium hydroxide. The alkaline filtrate from the barium sulphate contained 405 mg. of iodine, 20 % of the iodine thus having been sacrificed at the stage of lead precipitation for reasons already given. The alkaline filtrate was combined with solution B (see above), and the whole, containing in all 523 mg. of iodine, was boiled down to 500 cc.; 200 g. of crystalline barium hydroxide were added, and the solution was heated for 18 hours at 100°. A small greenish precipitate was filtered off hot, but contained no significant amount of iodine; the filtrate (512 mg. of iodine) was cooled, the barium hydroxide was filtered off and recrystallised; the combined mother liquors were treated with carbon dioxide and the barium carbonate removed by filtration and well washed; the filtrate and washings contained 512 mg. of iodine. Silver nitrate (20 % solution) was now added until precipitation was complete, the mother liquor being free from iodine; the silver salts were filtered off and ground up with dilute nitric acid (free from nitrous acid); the filtered solution contained 328 mg. of iodine, indicating a loss at this stage of 184 mg. as iodide. The organic silver salts were re-precipitated by the careful addition of ammonia, filtered off, washed, suspended in water and decomposed with hydrogen sulphide; the solution was brought to the boil, the silver sulphide removed by filtration, and the filtrate, containing 305 mg. of iodine, was concentrated to 200 cc. under diminished pressure. The solution was now extracted by shaking out nine times with

butyl alchohol (previously purified by agitation with saturated sodium bisulphite followed by distillation) at a temperature of about 70°; the combined butyl alcohol extracts were evaporated to dryness under diminished pressure and the residue dissolved in water; the solution contained 284 mg. of iodine; the volume was made up to 500 cc., the solution was brought to the boil and treated with uranium acetate solution in slight excess; the precipitate was filtered off and the filtrate freed from uranium with ammonia and concentrated to 320 cc. under diminished pressure; it now contained 220 mg. of iodine; basic lead acetate solution was added to complete precipitation, and after standing overnight the precipitate was collected, well washed, and decomposed by saturating its suspension in hot water with hydrogen sulphide; the lead sulphide was boiled out with much hot water, and the filtrate and washings were concentrated to a small volume under diminished pressure; the solution, which was faintly acid to Congo red, was exactly neutralised to litmus with ammonia; on further concentration in a vacuum desiccator over sulphuric acid 225 mg. of a crystalline compound separated out. On recrystallisation from 50 % acetic acid it formed pale straw-coloured prismatic needles having M.P. 198·4° (decomp.); a sample of *dl*-3 : 5-diiodotyrosine, prepared by iodination of *dl*-tyrosine, had M.P. 197·5°, whilst a mixture of the natural and synthetic products melted at 198·0°[1]. The product from the thyroid gave the colour reaction with nitrous acid and ammonia with intensity; on evaporation of a small amount on the water-bath with concentrated hydriodic acid, a residue was left which gave a strong Millon's reaction.

Analysis. 2·168 mg. required 5·00 cc. *N*/200 thiosulphate [Kendall, 1914]
20·3 mg. gave 0·577 mg. N (Micro-Kjeldahl)
20·3 mg. gave 1·035 cc. moist N_2 at 21·5° and 764 mm.

	I	N
Found:	58·2	2·8, 2·9
Calc. for $C_9H_9O_3NI_2$:	58·6	3·2

There remained, therefore, no doubt as to the identity of the compound isolated from the thyroid gland with *dl*-3 : 5-diiodotyrosine.

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[1] The melting point of *dl*-3 : 5-diiodotyrosine is variously reported in the literature, values from 190° to 213° being given; using synthetic samples of indubitable purity, and heating fairly rapidly, we have never found it to lie above 199°.

XLVI. OBSERVATIONS ON INSULIN.

PART I. CHEMICAL OBSERVATIONS.

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PART I.

Since the first announcement by Abel [1926] of the isolation from commercial insulin of a crystalline substance exhibiting in a high degree the specific activity of the hormone, it has become a matter of the greatest interest to attempt to determine whether this crystalline material is indeed to be regarded as insulin itself, or whether it is in reality an inert constituent of the pancreas which, in the process of preparation, has become associated with the hormone by adsorption or otherwise. The final proof of the identity of the compound with insulin, if such identity exists, can naturally only be afforded by synthesis; since, however, we have to do with a substance which is to be classed among the simpler proteins the prospects of such a synthesis remain infinitely remote, and other, less direct, evidence bearing upon the question must be sought. Later papers from Abel's laboratory [Abel *et al.*, 1927; Jensen and Geiling, 1928; du Vigneaud *et al.*, 1928] have not increased the volume of such evidence to any considerable extent. Slight modifications have been introduced into the original method of preparation of the crystalline material, but these modifications do not successfully meet the criticisms which may be levelled at the claim to designate the substance pure insulin. In particular have the workers in Abel's laboratory failed to meet the criticism based on the varying degree of physiological activity reported for different samples of a supposedly uniform substance. If we turn to the experience of other workers with this material we find that a similar state of affairs seems to exist, for whilst there is no doubt that crystals identical with those

described by Abel have been prepared in other laboratories by his method, no consistency is to be found in the degree of physiological activity assigned to them by different workers, or indeed sometimes by the same worker. It has even been stated [Dingemanse, 1928] that it is possible by adsorption on charcoal followed by elution with phenol and extraction of the material recovered from phenol with phosphate to obtain an amorphous preparation of a higher degree of activity than that ascribed by Abel to the crystals; this statement is, however, contested by the workers in Abel's laboratory [du Vigneaud *et al.*, 1928].

The present uncertain position of the problem seemed to us to warrant a renewed attempt to obtain more decisive evidence upon the point at issue, and we therefore set out on the present investigation with three main objects in view: (*a*) to obtain the material by Abel's method and to subject it to thorough physiological assay, (*b*) to seek for alternative methods of preparation, and (*c*) to carry out certain experiments with the crystalline substance designed to test the question of its identity with insulin.

As starting material in this work we have employed in the main two commercial preparations of insulin. The earlier experiments were made with a powder prepared at the Connaught Laboratories in Toronto and assayed at 13 international units per mg., and the later experiments with a powder of approximately similar unitage kindly supplied to us by the British Drug Houses, to whom, and in particular to Dr F. H. Carr, we wish here to express our best thanks. We have also had the opportunity to make a few experiments with a powder prepared by Messrs Burroughs Wellcome and assayed at 21 international units per mg., for which we are indebted to Dr H. A. D. Jowett. Our first experiments consisted in attempts to prepare crystalline material from the Toronto insulin by the method described by Abel. After several failures, we were eventually able to obtain a small amount of the crystals by this method; our experience with the technique was discouraging, however, since we found it necessary to carry out many more reprecipitations than are indicated by Abel, with the result that the manipulation became excessively laborious and the yields of crystalline material were exceedingly small. As soon, therefore, as we had accumulated sufficient material for the purpose of physiological assay, we passed on to the second object of our investigation, namely the search for an alternative method of preparation.

The first impression which we, and apparently Abel himself, formed (although subsequent work has caused us to modify this view) was that the success of Abel's method depended on the efficient buffering achieved by the complex mixture of weak bases which he employed; it was thought that such a system enabled the reaction of the solution to be adjusted so gently and so exactly to the isoelectric point that it was possible in effect to obtain a supersaturated solution from which the protein might be expected to separate slowly, with consequently increased tendency to crystallise. With this idea in mind we sought to substitute a simple phosphate buffer for the mixture

of brucine acetate and pyridine employed by Abel, the insulin being dissolved at a slightly alkaline reaction, and the p_H of the solution adjusted by the very gradual addition of carbon dioxide; even the very slowest introduction of the latter, however, caused an immediate amorphous precipitate to form and by no fractionation on these lines were we able to obtain crystalline material. In the light of our subsequent experiments we are inclined to think that the function of the brucine acetate-pyridine mixture is not merely, as we first supposed, that of a delicate buffering system, but that other physical properties of these substances are involved, *e.g.* the lowering of the surface tension of the solution, thus bringing into play an effect on the properties of the commercial insulin analogous to that about to be described. Some such explanation of the rôle of brucine is indeed suggested by Abel himself.

The method for the preparation of the crystalline material which has met, in our hands, with a certain amount of success, and which we wish to describe in this paper, rests on the observation that the properties of commercial insulin are profoundly modified by the presence in the solution of an active saponin. As to what exactly constitutes an "active" saponin from this point of view we shall have more to say later; at this point let us assume that we are speaking of one which has been found by experiment to be satisfactory for the purpose in hand. It is well known that the commercial purification of insulin consists, in its final stages, of repeated precipitations at the isoelectric point, the latter lying at about p_H 4·8–5·0; a good commercial product, such as either of those which we have used in this work, precipitates practically quantitatively when the solution is adjusted to this reaction. If, however, a sufficient amount of saponin be introduced into the acid solution, the reaction may be carried, by the gradual addition of alkali, over the isoelectric point without the occurrence of any precipitation whatever. It might be thought at first sight that we have to do here merely with an instance of inhibition of precipitation or supersaturation such as is known to occur in simpler cases in the presence of saponin; that such a straightforward explanation fails to fit the facts is shown, however, by the effect of lower concentrations of saponin. The saponin possesses apparently the additional power of, so to speak, sharpening the isoelectric points of the proteins present. If a solution of insulin in dilute acetic acid containing about 1 % of saponin is treated gradually with ammonia, a definite turbidity is observed when the p_H is about 4·5; in the absence of saponin, turbidity occurs earlier and a gross flocculation is already apparent at this reaction. On keeping the saponin-containing solution, a flocculent precipitate separates in the course of about 30 minutes, and may be removed on the centrifuge, when it is found to represent about 30 % of the weight of the starting material but only 15–20 % of the activity. Whilst continued addition of ammonia to the solution of insulin in dilute acid in the absence of saponin causes steadily increasing precipitation up to about p_H 5·0, further addition of alkali to the saponin-containing solution after removal of the precipitate at p_H 4·5 causes no visible

change until the reaction reaches p_H 5·6, at which point an opalescence develops; if the addition of alkali be interrupted and the solution kept overnight a precipitate forms which, in a satisfactory experiment, is already partly crystalline. A second similar isoelectric precipitation from more concentrated solution in presence of saponin results in a product which is almost entirely free from amorphous material, although not yet of very definite form. The precipitate may now, however, be successfully recrystallised by isoelectric precipitation from a phosphate buffer solution without the aid of saponin, and, by the selection of appropriate conditions of temperature and concentration, may be obtained in remarkably large and well-defined crystals.

Given, therefore, a suitable saponin we possess, in the technique summarised in the last paragraph, a simple and rapid means for the preparation of crystalline material from a commercial insulin powder of the unitage indicated. There are many factors, however, which render the situation less simple than is indicated by the above brief description. The difficulties of standardising any method requiring the delicate adjustment of physico-chemical conditions, which involves the use of saponin, are obvious. Commercial saponins are crude substances of varying origin and variable properties; from the commercial point of view the value of a saponin is determined solely by its frothing capacity, and we were soon able to show that this property by itself fails to give an indication of the efficiency of a saponin for our purpose. We are indeed quite unable to offer any rational explanation of the mechanism of the action of saponin in promoting the crystallisation. That it is not, entirely at any rate, a question of the lowering of surface tension is indicated by the failure of substances such as the higher alcohols, which lower surface tension to a still greater extent, to achieve the same object, at least with a similar efficiency. Part of the effect is undoubtedly to be ascribed to the power of saponins to inhibit precipitation in general, and in so far as this is concerned, there is some indication that a high frothing capacity on the part of a saponin implies also a high capacity for promoting supersaturation. A not less important factor, however, is the apparent ability of certain saponins to effect the sharpening of the isoelectric points of the mixture of proteins which constitute commercial insulin, with the result that it becomes possible to precipitate the proteins individually at fairly widely differing and clearly defined points instead of throwing down the mixture *en masse* over a more extended range of p_H.[1] It is evident that from the preparative standpoint these two properties of the saponin are to some extent mutually antagonistic. The more saponin used the greater is the degree of supersaturation induced and hence the lower is the yield of crystals; on the other hand, the crystallisation fails entirely to occur if enough saponin be not used to effect the separation of the isoelectric points (if we may so designate the phenomenon discussed above) in a well-defined manner. By a fortunate coincidence the first sample of saponin which we tried possessed eminently satisfactory properties in both respects. The next sample, although ostensibly

of similar quality, was almost entirely useless for our purpose; various subsequent samples of saponin have exhibited differing degrees of efficiency, as determined by the number of precipitations necessary for the purification and by the yield of crystalline product obtained. The cruder samples contained considerable amounts of calcium sulphate and traces of heavy metals; with such samples, as might be expected, immediate precipitation of part of the insulin occurred even in fairly acid solutions, and an unduly large amount of material was therefore removed with the first acid precipitate at p_H 4·5. Apart from the elimination of such gross impurities, however, it was difficult to detect any distinguishing feature by which a saponin might be recognised as being suitable for the purpose in hand. In the search for such a criterion we investigated the haemolytic power of the various saponins employed, for help in which part of the work we are indebted to Dr H. D. Wright; as will be seen from the results recorded below there does appear to exist at least a rough parallelism between the haemolytic indices of the different saponins and their efficiencies in promoting the crystallisation; as an approximation it may be said that, as far as our experience extends, a saponin with a haemolytic index of less than 1 : 8000 is unlikely to be of much use for the purpose.

Apart from the variability of the different saponins themselves, a further complication is introduced by the effect of surface. The method was first worked out for 20 mg. samples of the crude insulin powder, and we were for a long time troubled by our inability to extend the process to larger amounts. Thinking that the difficulty might be due to the importance of preserving correct time-relationships between the different stages of the manipulation, we performed many experiments on the adjustment of these relationships, all however to no purpose. We were then reminded of the fact that when a solution of saponin is introduced into a glass vessel a gradual concentration of the saponin occurs at the liquid-glass interface, and this was apparently the key of the situation. In the earlier small-scale experiments mentioned above the solution was always transferred, at the crystallising point, to 15 cc. centrifuge cups in which the liquid-glass interface was about 25 cm.^{2} for a total volume of 9 cc.; in the larger experiments involving greater volumes of liquid, the final solution was left in a beaker, or transferred to larger centrifuge cups of a different shape; in either case the ratio of liquid-glass interfacial area to volume of solution was significantly lower than that in the small experiments; in the case of the beaker the surface was only 45 cm.^{2} for a volume of 45 cc. Such larger experiments led to no good result, and it was only when, after many variations, we restored this ratio to its original value by dividing the final solution, as soon as it was adjusted to the correct p_H over a large number of the 15 cc. cups, that we were successful in achieving the crystallisation of larger amounts. We were subsequently able to bring about the same result by leaving the solution in the beaker and introducing the requisite number of glass rods to bring the interfacial area to the same proportionate amount as in the small tubes. It may be noted here that it is

in the first separation from the crude insulin powder that the surface relationship is of paramount importance; once the precipitate has been obtained even partly crystalline this factor becomes of less significance. In all probability there exists an optimum relationship between the concentration of saponin employed and the surface-volume ratio which we have just discussed, and the particular suitability of the 15 cc. centrifuge cups for our purpose is decided by the concentrations of saponin which we have arbitrarily selected. Much further experimental work, however, will be required to clear up this point, and, for the present, we have contented ourselves with the preservation of the correct relationship by the simple expedient indicated, since we have not attempted to extend the method to larger quantities than 0·5 g. of the crude powder. It is hoped in the near future to carry out further experiments with the object of defining the optimum conditions more exactly. In order to make the method, at least for small quantities, readily reproducible, we have worked out the details for the crystallisation using pure digitonin instead of a commercial saponin; the conditions for the use of ordinary saponins can at present only be described in somewhat general terms, the exact details having to be worked out for each sample.

Perhaps the least satisfactory feature of the whole method is the poorness of the yield. With the different saponins of which we have had experience this has varied from 5 to 15 % of the crude powder, which, considering the relatively small access in activity on crystallisation, evidently leaves much to be desired; we have, however, done little or nothing towards working up residues, and it is not unlikely that a systematic application to this task would effect a considerable increase in the total yield obtainable.

It will be apparent from what has been said that a large amount of experimental work remains to be done in order to realise to the full the possibilities of the method which we describe. Nevertheless we are publishing our results at the present stage, partly because our immediate association in the work can unfortunately not be continued, but also because we feel that, even so far as our experiments have gone, we have some evidence to offer bearing on the question of the identity or non-identity of the crystalline compound with insulin.

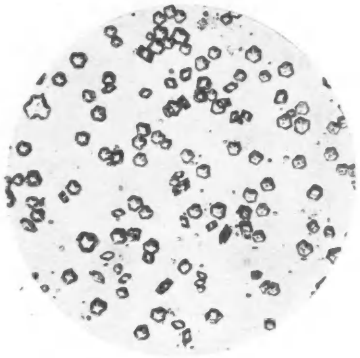
It may be said at once that the strongest evidence on the last-mentioned point is to be found in the results of the physiological assay contained in Part II of this paper. The impressive consistency of the results reported by independent workers for the activity of four different samples of crystals, two obtained by Abel's method, and two by our own, must, we think, be accepted as a powerful argument in favour of the identity of the compound with insulin. In addition to the results recorded in Part II assays were performed by the mouse method on samples of crystals prepared from the B.D.H. insulin by the use of saponin and from the insulin of Messrs Burroughs Wellcome by the aid of digitonin. In both cases the product showed an activity of 25 international units per mg.

To obtain evidence as impressive from the chemical side is a difficult matter, but there is no doubt that the results of our experiments point in the same direction. The crystals themselves exhibit a clearly defined structure, which is well depicted in the photograph (Plate I) (kindly taken for us by Mr J. E. Barnard); all preparations present an appearance of absolute uniformity, not only to our eyes, but to those of a crystallographer[1]; the possibility that they may represent a mixed crystal containing insulin as one component seems therefore remote. There remains the more likely suggestion that they may owe their physiological activity to the adsorption of a trace of a highly active substance, and this criticism is the more difficult to meet, since the crystals can only be obtained under one set of physico-chemical conditions, namely by isoelectric precipitation. We think, however, that the success of the saponin method does afford some evidence against the adsorption theory, since it is difficult to suppose that the conditions of adsorption would not be modified by the presence in the solution of so highly surface-active a substance as saponin, and yet the saponin method and that of Abel lead to products of identical activity. Further, we have been able to show, in confirmation of Abel, that the crystallisation may be effected from 50 % alcohol instead of from water without modification of the activity of the product. Negative evidence on the same point is provided by the failure of our attempts to recover any crystalline material from solutions of the crystals which had been subjected to the mildest chemical treatment consistent with destruction of the physiological activity.

The conception that insulin, with its high degree of specific physiological activity, should be a protein without any obvious distinguishing chemical property is admittedly not readily acceptable; still less acceptable to many minds will be the notion that the best commercial products, prepared by empirical methods, should represent almost pure insulin. The latter objection is, however, based on prejudice rather than on reason, and we suggest that both difficulties may be largely removed by a hypothesis which we wish tentatively to advance.

Let us suppose that we take thyroid gland, extract therefrom the iodothyreoglobulin, and, by some manipulation, succeed in obtaining the latter in the crystalline form. We should then have in our hands a crystalline protein exhibiting (although, owing to its large molecular weight, not in a very high degree) the specific physiological activity of the gland; we might, therefore, not unjustifiably, designate the product the pure active principle of the thyroid. In the light of our present knowledge we recognise that such a designation would be correct only in a limited sense, since the actual physiological activity is due, not to the protein as a whole, but to its specific constituent thyroxine. In a similarly limited sense we are inclined to regard the crystalline protein

[1] The crystals were kindly examined for us by Dr T. V. Barker of the Department of Mineralogy, University of Oxford, who expressed the opinion that the preparation had the appearance of "a definite substance with well-defined crystallophysical properties." The crystals were too small to permit of complete crystallographic measurements.



General field of crystals. ×120

Single crystal. ×340

Stereoscopic photograph of single crystal. ×340

PHOTOMICROGRAPHS OF INSULIN CRYSTALS

from the pancreas as being the true active principle of the gland in so far as the function of the latter in relation to carbohydrate metabolism is concerned. We feel that in this case, as in that of iodothyreoglobulin, the physiological activity is a property, not of the protein as a whole, but of some specific constituent or grouping contained within its molecule. The thyroid protein is differentiated from other proteins in an obvious manner by its iodine content, and the specific constituent, being a simple amino-acid, can be readily separated from the rest of the molecule; the insulin protein bears no such chemical earmark, unless it be the high content of sulphur. The analogy which we have drawn between the thyroid protein and the insulin protein must not be taken to indicate that we believe such a separation of the specific group to be necessarily practicable in the latter case. As a theoretical possibility, however, it seems worth bearing in mind.

Experimental.

The method of Abel. The first experiments consisted in attempts to apply the method described by Abel *et al.* [1927] to the preparation of crystalline material from the Toronto insulin powder assayed at 13 international units per mg. Although we followed the directions given by Abel with the greatest care, all our solutions being carefully checked, we met at the outset with a puzzling discrepancy in that we failed to obtain any precipitate on the addition of pyridine. In this respect the crude insulin was apparently behaving in a manner similar to that noted by Abel as characteristic of the crystalline material. We, therefore, proceeded with the addition of ammonia, using a 0·85 % solution as indicated by du Vigneaud *et al.* [1928][1]; after addition of the specified amount of ammonia we obtained a precipitate which was removed on the centrifuge, forming a somewhat gummy mass; nothing separated from the mother liquor on keeping the latter in the ice-chest, and, on testing, the solution was found to be considerably more acid than it should be according to Abel's description; further ammonia was added (3 cc. for every 0·1 g. of original insulin) to bring the reaction[2] of the solution to p_H 5·6. At this point an opalescence developed, and, on keeping the solution in the cold, a precipitate settled out, which was, however, amorphous. By repeatedly putting this precipitate through a similar process in continually diminishing volume (using always more ammonia than indicated by Abel) the material was eventually obtained in the crystalline condition as described by Abel, and was recrystallised in the absence of brucine either by the method given in his paper, or better from a phosphate buffer as described below. No explanation of the differences observed by us can at present be given. We mention our experience for the possible interest of other workers who may

[1] In the paper by Abel *et al.* [1927] the use of 0·65 % ammonia for the crystallisation is described; a subsequent paper by du Vigneaud *et al.* [1928] from Abel's laboratory gives 0·85 % as the concentration of this reagent, no reason for the change being given so far as we can ascertain.

[2] All p_H determinations mentioned in this paper were made colorimetrically.

be attempting to carry out this manipulation. The discrepancy was not due to excess of acid adherent to or combined with the original insulin, since this was neutralised at the outset; attempts at purification of the crude powder by the phenol treatment of Abel or by continuous extraction of an isoelectric suspension with butyl alcohol under diminished pressure failed to bring the behaviour of the insulin into line with that of the sample which Abel employed, although the butyl alcohol extraction, which removed a small amount of physiologically inert material (possibly of a lipoid character) did appear to improve the conditions for crystallisation to a small extent. At the best, however, the yields were so minute and the method so time-consuming that we soon passed on to the attempt to find an alternative procedure, particularly as we were anxious to break away entirely from the use of brucine—a substance which, in a case such as the present, has very obvious disadvantages.

Experiments with carbon dioxide. It was thought that the reaction of an alkaline buffer solution containing insulin might be adjusted so delicately by the gradual introduction of carbon dioxide that we might in this way achieve the necessary supersaturation at the isoelectric point, dispensing with the elaborate mixture of weak bases. With this object 0·1 g. of insulin was dissolved in 4 cc. of water together with 0·3 cc. of 0·1 *N* hydrochloric acid, and this solution was mixed with 10 cc. of a phosphate buffer at p_H 7·0 to which had been added 1·5 cc. of 0·1 *N* sodium hydroxide. A slow stream of carbon dioxide was passed into the solution through a capillary tube; after 3 minutes an opalescence was apparent. The addition of carbon dioxide was interrupted and the solution allowed to stand for some hours; the precipitate was separated on the centrifuge, and the mother liquor further treated with carbon dioxide; this caused almost immediate further precipitation, and by the time the reaction had reached about p_H 6·4 the greater part of the insulin had separated in the amorphous condition; no tendency to crystallise was observed at any stage, nor did any fraction obtained by precipitation with carbon dioxide appear to be more suitable for crystallisation by the brucine method than the original crude powder. These experiments were, therefore, not pursued further.

Saponin method. The observations on which this method is based have been discussed in the introductory part of this paper and need not be further referred to here; nor shall we do more than mention the very numerous experiments which have been made on the effect of variations in temperature and in the time-relations of different parts of the process, and on the optimum point at which to remove the first precipitate. In this section we propose to describe (*a*) the method by which we gauge the most suitable amount of saponin to employ, (*b*) the crystallisation with the aid of a good commercial saponin, (*c*) the method with the use of digitonin.

(*a*) *Determination of correct amount of saponin.* A 6 % solution of the saponin in distilled water is made. (This solution should be prepared freshly

each day owing to the rapidity with which it becomes infected.) A stock solution of insulin is then prepared by dissolving 0·1 g. of the commercial powder in 5 cc. of 10 % acetic acid and making the volume up to 25 cc. with distilled water. This stock solution is divided into five equal portions, and these are treated with 0·5, 0·75, 1·0, 1·5 and 2·0 cc. of the saponin solution respectively. The solutions are warmed to 35° and to each portion is added 1·6 cc. of 0·85 % ammonia. After standing for 30 minutes at 35° the precipitates which have formed are removed on the centrifuge, and the mother liquors are adjusted to p_H 5·6 by the addition of a further 1·7 cc. of the 0·85 % ammonia and transferred at once to 15 cc. centrifuge cups. It is important to adjust the reaction to p_H 5·6 as rapidly and as sharply as possible; the exact amount of ammonia necessary will vary slightly with different samples of insulin according to the amount of acid present. The tubes are allowed to stand, with occasional scratching with a glass rod, and are examined next day. With a good saponin the general result of such an experiment will be that in the tubes with the lower concentrations of saponin there is a large amount of amorphous precipitate whilst in those with the high concentrations the precipitate is mostly crystalline but small in amount; it is usually fairly easy to pick out that tube of the series in which conditions are best, *i.e.* where there is the largest amount of precipitation consistent with the absence of grossly amorphous material. It must be understood that, in speaking of crystalline material at this stage we do not mean to indicate that it already has a well-defined form; this is not the case, but with some experience it becomes quite easy, on examination under a high power of the microscope, to distinguish between material which, while not yet of definite form, will recrystallise satisfactorily, and the ragged grossly flocculent impurities which must be avoided. It is really only by experience that these differences can be recognised; a more precise verbal description is difficult to give. However, those tubes in which conditions are good are usually macroscopically distinguished by the separation of material on the sides of the vessel where it has been scratched with the glass rod. In the case of our best commercial saponin optimum conditions obtained in the tube to which 1·0 cc. of the saponin solution had been added; in this case, therefore, a typical experiment was as follows.

(*b*) *Crystallisation with commercial saponin.* To 5 cc. of the stock solution of insulin in 2 % acetic acid (representing 20 mg. of the crude powder) was added 1 cc. of 6 % saponin solution; the mixture was warmed to 35°, treated with 1·6 cc. of 0·85 % ammonia, and kept at 35° for 30 minutes; the precipitate was removed on the centrifuge and the mother liquor adjusted as rapidly and sharply as possible to p_H 5·6 by the addition of 1·7 cc. more of the ammonia, and transferred immediately to a 15 cc. centrifuge cup. At this point the solution was still clear or only very slightly opalescent; on standing and scratching with a glass rod a turbidity soon developed, and overnight a precipitate separated which was largely micro-crystalline in

character. Five such tubes were usually prepared simultaneously, or 100 mg. of the crude powder was worked up as a whole, the final solution being divided over 4 or 5 tubes; after separation was complete the precipitates were collected with the aid of the centrifuge and combined in solution in 2·5 cc. of 10 % acetic acid and 10 cc. of water; 2·5 cc. of the 6 % saponin were then added and the reaction adjusted immediately to p_H 5·6 by the addition of 8·25 cc. of 0·85 % ammonia. This time the precipitate was almost entirely microcrystalline, although not yet of definite form. A further crop of crystalline material could sometimes be obtained by saturating the mother liquor with butyl alcohol. The final crystallisation was effected as follows. The whole of the precipitate after the second separation in presence of saponin (representing 100 mg. of crude insulin) was dissolved in 4 cc. of water and 0·4 cc. of 0·1 *N* hydrochloric acid; the solution was poured with stirring into a mixture of 16 cc. of a phosphate buffer at p_H 7, prepared according to Clark [1923], to which had been added 0·8 cc. of 0·1 *N* sodium hydroxide; the resulting solution was warmed to about 55°, treated with 1·0 cc. of 0·1 *N* hydrochloric acid, which brought the reaction to p_H 5·6, and set aside. Within a few hours there separated a precipitate which was now wholly crystalline and exhibited the characteristic form. The size of the crystals could be increased by one or two further separations from phosphate solution on the same lines. The best results in this last crystallisation were obtained if the solution, after adjustment to the isoelectric point, were left entirely undisturbed so that the material should separate as slowly as possible. The yield varied with the saponin and the crude insulin employed, amounting to 5–15 % of the starting material. A physiological assay in one case indicated that 50–60 % of the activity remained in the combined mother liquors, 15–20 % being lost in the first acid precipitate, which however amounted to 30 % of the weight of the original insulin; this partition probably varies somewhat with different saponins and different preparations of insulin. We have, up to the present, made no attempt to subject the material recovered from the mother liquor to crystallisation; that it would almost certainly be possible to do so with more or less success is indicated, however, by an experiment in which we were able to obtain crystals from a crude powder of as low an activity as 8 international units per mg.

We have had experience in all of nine samples of saponin of which the first was considerably the best; this was a sample of "white saponin" purchased from the British Drug Houses. The second sample, purchased under the same description, turned out to be practically useless; subsequently we tried a number of commercial samples, all of which were more or less useful for our purpose, although some of the cruder ones, containing as they did large amounts of inorganic matter, even including traces of heavy metals, caused an apparent denaturation of a part of the insulin. In the attempt to find some correlation between a measurable physical property of a saponin and its usefulness for our purpose we have compared in Table I the frothing

capacity and haemolytic power of the various saponins with their efficiency in promoting crystallisation. In this table the frothing number is given as that concentration which on shaking for 15 seconds in a tube of 16 mm. diameter produces 1 cm. of froth measured after 15 minutes. The haemolytic power is expressed as the minimum concentration necessary to produce complete haemolysis of a 5 % suspension of rabbit's washed red blood-corpuscles in 0·9 % sodium chloride during 2 hours at 37°. (For these determinations we are indebted to Dr H. D. Wright.) Unfortunately the data are not available for the first and best of our saponins, since we had exhausted the supply of this material before running into the difficulties which persuaded us to make the comparison. The true haemolytic value for digitonin should probably be somewhat higher than that given, since part of the digitonin is precipitated, presumably by the cholesterol from the cells, and is thus removed from the solution. The general result of the comparison is to indicate that, from the point of view of efficiency in promoting the crystallisation, the haemolytic index of a saponin is of more significance than is its frothing power.

Table I.

Saponin	Frothing number	Haemolytic index	Crystallising efficiency
White No. 1	Not determined	Not determined	+ + +
" " 2	1 : 25,000	1 : 4,000	Useless
" " 3	1 : 25,000	1 : 8,000	+ +
A	1 : 20,000	1 : 32,000	+ +
B	1 : 16,000	1 : 32,000	+ +
C	1 : 15,000	1 : 32,000	+ +
D	1 : 15,000	1 : 32,000	+
E	1 : 9,000	1 : 16,000	+
F	1 : 10,000	1 : 32,000	+ +
Digitonin	1 : 30,000	1 : 8,000	+ +

Digitonin method. The insulin powder (0·1 g.) is dissolved in 5 cc. of 10 % acetic acid and the solution is made up to 25 cc. with water; 0·125 g. of digitonin dissolved in 2·5 cc. of water is now added, followed by 8 cc. of 0·85 % ammonia; after standing 30 minutes, the precipitate is removed on the centrifuge and the mother liquor adjusted to p_H 5·6 by the addition of 8·5 cc. of 0·85 % ammonia; the solution is then divided over four or five 15 cc. centrifuge cups and set aside; after standing for some hours (preferably overnight) the precipitate is separated on the centrifuge and subjected to a similar process, using half the quantities of all reagents; after the second separation the precipitate may be successfully crystallised from a phosphate solution exactly as described above. In the case of the digitonin, as in those of some of the commercial saponins, there may be little sign of actual crystallisation as the result of the first two precipitations, the visible improvement consisting rather in the elimination of the grossly flocculent amorphous material; so long as the removal of the latter is complete, however, the final crystallisation from the phosphate solution succeeds. The yield by the digitonin method has not been more than 10 % of the starting material.

Miscellaneous experiments.

(*a*) *Butyl alcohol.* The observation noted above that a further crop of crystalline material may be obtained from saponin-containing mother liquors, combined with the consideration of the part which must be played in the whole process by the lowering of the surface tension of the solution produced by saponin, led us to try some experiments on the effect of butyl alcohol alone. Crude insulin was, therefore, subjected to a process similar to the phosphate crystallisation described above except that 5 % of purified *iso*butyl alcohol was added to the alkaline solution immediately before adjustment to the isoelectric point. In this way we were indeed successful in obtaining some material which appeared partly crystalline, but attempts at recrystallisation did not lead to a satisfactory product. It was, moreover, noticed that part of the material had lost its acid-solubility as the result of the butyl alcohol treatment, *i.e.* was partly denatured, and these experiments were, therefore, not pursued further.

(*b*) *Bile salts.* In the search for a substitute for saponin of a more reproducible character than the latter, an experiment was made to see whether bile salts could be employed; it was found, as was anticipated, that they were useless in solutions of the acidity which we were employing.

(*c*) *Inactivation experiments.* In order to obtain some evidence bearing on the question of the identity of the crystalline material with insulin, particularly in relation to the adsorption theory discussed in the introductory part, attempts were made to recover crystals from solutions of the latter which had been subjected to the minimum treatment with alkali which was necessary to bring about more than 80 % inactivation. The crystals (20 mg.) were dissolved in an alkaline phosphate solution (40 cc.), the p_H being approximately 10·5; in order to produce the required degree of inactivation, this solution had to be heated at 70° for 30 minutes; after such treatment, on readjustment to p_H 5·6, only amorphous material separated, nor could any crystals be obtained by subjecting this material to the saponin treatment. The inactivation by alkali was apparently irreversible, *i.e.* no regeneration of activity or of potentially crystalline material was observed on making the reaction acid and keeping the solution.

Appearance and analysis of the crystals.

The preparations of crystalline material present always an appearance of absolute uniformity. Seen under a low power the most conspicuous feature is the hexagonal outline noted by Abel. Examined more closely under a high power the crystals present the appearance of cubes, or rhombohedra approximating to cubes, which are standing on one corner, thus giving rise to the hexagonal outline already mentioned. This description has naturally not the slightest crystallographic significance, and the crystals are too small for complete angular measurements to be made. They are weakly doubly-refracting and have a refractive index of approximately 1·58. The optical appearance of

the crystals is better depicted in the accompanying photographs (Plate I) than it can be described in words.

For analysis, some of the material was dried in a vacuum desiccator over sulphuric acid at the ordinary temperature and then allowed to come to equilibrium with the air; analysed in this condition it gave the following figures, which agree closely with those given by Abel for his air-dried substance[1].

4·861 mg.	gave	8·79 mg.	CO_2, 2·9 mg. H_2O
4·960	„	8·93 mg.	CO_2, 3·08 mg. H_2O
2·945	„	0·365 cc.	N_2 at 21·5° and 767 mm.
3·028	„	0·364 cc.	N_2 at 22° and 767 mm.
11·200	„	2·535 mg.	$BaSO_4$

Whence	C	H	N	S
	49·61	6·81	14·49	3·11
	49·11	6·95	14·03	—

PART II.

In Part I of this paper is described the preparation from insulin of crystals, indistinguishable from those obtained by Abel and his colleagues, by the aid of a simpler method than that which they have used. Preliminary trials showed that these crystals possessed the specific activity of insulin in a high degree. It appeared to be important to have this physiological activity measured as accurately as the available methods allow. Two questions concerning the activity of such insulin crystals seemed to us to require more definite answers than they have hitherto received.

1. Do the crystals, apparently pure and identical, obtained by different procedures, show a uniform activity, or an activity which varies from batch to batch? A precise answer to this question would have an obvious bearing on the claim of the crystals to be regarded as insulin itself in pure form. A wide variation in activity from batch to batch would be inconsistent with such an identification; uniform activity, on the other hand, in batches prepared by different methods from different samples of the crude insulin, while not affording conclusive proof that the crystals are pure insulin, would be difficult to reconcile with the view that they consist of a non-specific, inert material, on which the insulin is merely adsorbed.

2. If the crystals show uniform activity, what is the degree of that activity, expressed in the commonly accepted units?

In spite of the large amount of work on the subject during the past two years, the information on these two points, obtainable from the literature, is curiously incomplete and unsatisfactory. In his first paper on the subject Abel [1926] did not attempt to assign a definite unitage to the crystals, merely indicating that 1/100 mg. in one experiment, and 1/125 in another, had sufficed to reduce the blood-sugar of certain rabbits to the traditional

[1] Analyses performed by Dr Ing. A. Schoeller, Berlin-Schmargendorf, Tölzerstrasse 19.

"convulsant level" of 45 mg. per 100 cc. and even to produce actual convulsions. In a later paper, published with Geiling, Rouiller, Bell and Wintersteiner [1927], he assigned a value of 90 units per mg. to a sample of the crystals. More recently du Vigneaud, Geiling and Eddy [1928] have recorded a value of 54 units per mg., and Jensen and Geiling [1928] one of 45 units per mg., for different samples of the crystalline product[1].

Taken at their face value, these estimates do not give an impression of uniform activity in the different samples; nor, with estimates spread over so wide a range, is it possible to select one as representing the true and characteristic activity of the crystals; though the fact that actual convulsions are recorded in certain rabbits, receiving as little as 0·01 mg., suggests an activity higher than that of the purest insulin obtained by ordinary methods. On none of these points, however, can we regard the evidence of these measurements as satisfactory or convincing, since they were all obtained by the old method of direct calculation from the degrees of hypoglycaemia produced in unstandardised rabbits. It is not possible to attach a precise significance to unit values which have not been measured by comparison with, and expressed in terms of the International Standard, which since 1925 has afforded the basis for the unit by international agreement and general use. Such measurements of the activity of the insulin crystals, in relation to the International Standard, have been published by Freudenberg and Dirscherl [1928], who obtained the values of 23 and 26 units by different comparative methods. The difference between these two figures is not greater than the error of any method yet available; but the potency which they indicate is only about one-half of those more recently published from Abel's laboratory.

Plan of the investigation.

Four lots of the crystals were selected, two prepared by Abel's method, and two by the new method described in Part I. Taking them in the order in which they were examined they were as follows.

A. Crystals obtained by Harington and Scott, using Abel's method, from Toronto insulin having a unit value of 13–15 units per mg.

Test solution A made by dissolving 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

B. Crystals from the same Toronto insulin, prepared by Harington and Scott's "saponin" method.

Test solution B contained 11·8 mg. in 50 cc., *i.e.* 0·236 mg. per cc.

C. Prepared from the same raw material, and by the same method as B, but on a different occasion.

Test solution C contained 10·2 mg. in 50 cc., *i.e.* 0·204 mg. per cc.

[1] In a recent private communication to Dr Dale, Prof. Abel states that his colleagues are now obtaining values of 3 to 3½ times the activity of the International Standard (*i.e.* 24–28 units) for different samples of the crystals assayed in comparison with that Standard.

D. A mixture of two small lots of crystals kindly furnished by Prof. Abel, and prepared by his methods in his own laboratory.

Test solution D contained 8·2 mg. in 50 cc., *i.e.* 0·164 mg. per cc.

It was decided that each should be assayed independently by the four authors of this part, each of whom has long familiarity with the standardisation of insulin for practical use. Two different types of method were used. Scott and Trevan used the essentially statistical method, based on the relative frequencies of the appearance of hypoglycaemic convulsions in a large number of mice injected with a dose of the preparation under test, and in an equal number of similar mice injected at the same time with a control dose of the standard preparation, and observed under identical conditions. The method was independently elaborated, and with some differences of detail, by Trevan and Boock [1926], and by Krogh [1926]. Both Marks and Culhane used the method based by Marks [1925, 1926] on parallel observations of the degree of hypoglycaemia produced, by a dose of the preparation under test and by a dose of the standard preparation, in the same series of rabbits on different occasions; changes in the sensitiveness of the whole series being eliminated by injecting half the number with each preparation on each occasion, and reversing the groups on the two days of the test. Variations in the details of the application of these tests are mentioned below.

So that no question should arise of differences in the weighing out or solution of the preparations, a sufficient quantity of solution for the tests of all four investigations was prepared by Dr Harington from each of the batches of crystals. Physiological saline, acidulated with HCl to p_H 2·5, was used for the solution of the crystals, and the solutions were not made up to identical strengths of the crystalline material. In each case a quantity in the neighbourhood of 10 mg., but varying in the four cases from 8·2 to 11·8 mg., was dissolved and made up to 50 cc.

A common stock solution of the International Standard Insulin was similarly prepared by Marks, who weighed out and dissolved the dry powder in distilled water, making a solution of acidity about p_H 3·5 which contained 2·5 mg. of the standard preparation, and therefore had an activity of 20 units per cc. For each test each of us received a sufficient quantity of the standard solution, containing 20 units per cc., and of the solution of one of the crystalline preparations, of which the concentration as well as the activity was unknown to him. It was understood, however, that, in order to save time and animals, the solutions of A, B, C and D would not differ widely in concentration.

Each of us completed the determinations on any one of the preparations, A, B, C and D, without consultation with the others. In each case we handed our estimates independently to Dr Harington or Dr Dale, and they were not divulged or submitted to us for comparison until all four had been deposited.

ESTIMATES BY D. A. SCOTT.

The experiments were carried out at the National Institute for Medical Research, with the equipment there available. The mouse method, as advocated by Krogh and by Trevan and Boock, was used. The details followed were mostly those given by Trevan and Boock [1926]. The thermostat bath, in use at the National Institute to maintain the temperature in the mouse-boxes during the experiment, follows the general lines of Trevan's description, but has a motor-driven stirrer, an electrically-controlled thermostat, and a convenient weighting of the boxes.

The method has been for some time in use in the Connaught Laboratories, University of Toronto, with the following departures in detail from Trevan and Boock's description.

1. Mice showing convulsions in the course of a test are restored by injection of glucose, and after a week the whole stock are found to be as suitable for a further test as a fresh stock of mice.

Krogh's experience and practice are here confirmed and followed.

2. The volume of solution injected into each mouse is 0·25 cc. According to our experience a larger injection volume entails danger of leakage. Since the mice used in any experiment are all of about the same weight, not varying more than 1 g. in either direction from the mean, the mean weight can be accepted for all, and the required dose per g. of standard or test solution made up so as to occupy 0·25 cc. per mouse.

3. Observation was completed in 1½ hours, in place of the 2 hours indicated by Trevan. Since about 90 % of the mice which show convulsions do so within the first hour, the time of observation could probably be made even shorter.

The mice used in these tests were all of weights between 19 and 21 g. All but a few were bred at the National Institute, and were from 2½ to 3 months old at the time of their first use for these tests. The diet of all consisted of white English oats and stale brown bread, the latter being moistened with water. The mice to be used in an experiment were transferred to an empty cage and deprived of food at 5 p.m. on the previous afternoon. The injections were begun at 10.30 a.m. Injections were subcutaneous, and were made with a convenient precision-syringe of 0·25 cc. capacity. Each mouse, after injection, was immediately transferred to its appropriate box in the thermostat bath, which was kept throughout the experiment at 37·5–38°. The mice were kept as quiet as possible, and the number showing actual convulsions, or equivalent symptoms of collapse, was recorded. Trevan's test, depending on the inability of a mouse to right itself immediately, when placed on its back, was found useful in the recognition of symptoms "equivalent" to convulsions. Such mice, when the reaction was established, were removed and given a hypodermic injection of 0·2 cc. of 20 % glucose. At the end of 1½ hours the score was regarded as completed, and the mice which had not

shown definite "convulsions" or "symptoms" were removed, and food was given to the whole stock.

The determination of the unknown dose of the solution under test, from the relation between the proportions of convulsions produced by it and by the known dose of the standard, was made from the curve published by Trevan and Boock. After a preliminary orientating test, giving the approximate unit value of the unknown solution, an attempt was made in subsequent tests to give this and the standard in approximately equivalent dosage, and in each case to produce a convulsion-rate in the neighbourhood of 50 %. From the actual rates observed the true dose can then be read off the curve with a relatively small error.

The protocols are given at the end of the next section. From these it is seen that the figures obtained range from 24·2 units per mg. for D to 25·35 units for C.

Estimates by J. W. Trevan.

The tests were carried out by the mouse method as originally described by Trevan and Boock [1926], with the modification that the weights of the mice used were matched for the two solutions under test. Mice of 15–18 g. were used for one set of tests, mice from 18 to 25 g. (a very few being over 25) in another. None was used below 15 g. We have taken this precaution for some time.

The protocols are given at the end of this section. The figures in the last column of each table are calculated from the standard curve given in the original description of the method. The values for tests 1 and 2 on A are calculated from convulsion rates which are too low for much reliance to be placed on the calculated potency, although the results do agree with the later ones. However, the deduction can be made that the characteristic for insulin A is not significantly different from that of the International Standard given in the paper mentioned above.

One test (No. 5) on A was made, in which only low dilutions were used, and minute doses both of the standard and of A were injected by means of micrometer syringes. This was done to check whether in high dilutions the purer insulin was destroyed by surface action or shaking. No significant difference was found.

From the protocols, it is seen that the average figures vary between 22·8 units per mg. for C and 25·8 units for B.

Discussion of mouse test results.

The results of the mouse test by both workers are collected and compared in Table I. The degree of uncertainty of the final results is represented by the columns headed "range of possible values." These figures are calculated from the shape of the mouse insulin characteristic already published, and from the number of mice used, by the use of the formula given by Trevan

Table I. *Combined results and limits of error of mouse tests.*

	Scott		Trevan		
	Mean value (Units per mg.)	Calculated possible range	Mean value (Units per mg.)	Calculated possible range	Combined result
A	24·9	21·75–28·60	24·2	23·4–26·4	24·4
B	25·0	21·85–28·70	25·8	23·6–28·2	25·6
C	25·35	21·15–29·0	22·8	20·9–25·0	23·6
D	24·2	21·65–27·5	24·4	21·8–27·3	24·3
	Mean 24·8		Mean 24·3		Mean 24·5 (23·6–25·4)

[1929], assuming that the average convulsion rate is 50 %. This gives the error corresponding to approximately twice the standard deviation of the convulsion rate, and represents the range inside which the true value may be expected to fall with a probability of approximately 0·95. The discrepancy from the total average value of any single set of tests is not greater than might be expected to occur by errors of random sampling of mice. The most discrepant result is that of Trevan for C. The difference of this from the corresponding result by Scott on the same solution is, however, only 1·26 times its standard deviation. It is interesting that the individual values obtained for C by Trevan were much more concordant than those obtained

Protocols of mouse tests carried out by D. A. Scott.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
				Sample A.			
1	1/300	0·25 cc.	11/24	1/75	0·25 cc.	13/24	5·42
2	,,	,,	19/24	1/90	,,	15/24	5·05
3	,,	,,	18/24	1/75	,,	16/24	4·56
4	,,	,,	13/24	1/75	,,	15/24	5·42
						Mean	5·09
				Sample B.			
1	1/300	0·25 cc.	15/24	1/75	0·25 cc.	20/24	6·30
2	,,	,,	17/24	,,	,,	18/24	5·18
3	,,	,,	18/24	,,	,,	21/24	5·88
4	,,	,,	15/24	,,	,,	20/24	6·30
						Mean	5·9
				Sample C.			
1	1/400	0·25 cc.	20/24	1/100	0·25 cc.	19/24	4·72
2	,,	,,	16/24	,,	,,	18/24	5·45
3	,,	,,	17/24	,,	,,	20/24	5·70
4	,,	,,	16/24	,,	,,	15/24	4·80
						Mean	5·17
				Sample D.			
1	1/400	0·25 cc.	11/24	1/80	0·25 cc.	11/24	4·0
2	,,	,,	13/24	,,	,,	10/22	3·53
3	,,	,,	12/24	,,	,,	15/24	4·49
4	,,	,,	13/24	,,	,,	17/26	4·87
5	1/350	,,	15/24	1/70	,,	12/24	3·55
6	,,	,,	12/24	,,	,,	11/24	3·84
7	,,	,,	13/24	,,	,,	11/24	3·69
						Mean	3·97

for the other solutions, and this furnishes a good example of the uncertainty of relying on mere concordance of a limited number of observations as a guarantee of the accuracy of a result. The average result of the potency of all samples by the mouse test is 24·5 with a range of 23·6 to 25·4, the latter being worked out from the total number of mice used, namely, 2652.

Protocols of mouse tests carried out by J. W. Trevan.

	International standard 20 units per cc.			Solution of crystalline insulin			
Test No.	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Dilution	Amount injected per 20 g. mouse	Convulsion rate	Units per cc.
			Sample A.				
1	1/1000	0·5 cc.	25/30	1/1000	0·5 cc.	1/30	5·38
	,,	,,	19/30	,,	,,	0/30	
	,,	,,	21/29	,,	,,	0/29	
	,,	,,	19/30	,,	,,	1/30	
2	,,	,,	21/30	1/500	,,	2/30	4·37
	,,	,,	17/30	,,	,,	0/30	
	,,	,,	21/30	,,	,,	4/30	
3	1/1333	,,	19/30	,,	,,	15/30	6·61
4	,,	,,	18/30	1/400	,,	12/30	5·02
5	,,	,,	13/30	,,	,,	7/30	4·69
6	,,	,,	25/30	,,	,,	15/30	4·26
7	1/16	0·008 cc.	21/30	,,	0·008 cc.	24/30	5·75
8	1/100	0·5 cc.	24/30	1/250	0·5 cc.	16/30	3·82
9	,,	,,	18/30	,,	,,	10/30	4·68
10	,,	,,	15/30	,,	,,	10/30	4·17
11	,,	,,	16/30	,,	,,	18/30	5·33
				Mean omitting tests 1 and 2			4·92
				Mean including tests 1 and 2			4·92
			Sample B.				
1	1/1000	0·5 cc.	20/30	1/250	0·5 cc.	18/30	4·67
2	,,	,,	17/30	,,	,,	21/30	5·70
3	,,	,,	19/30	,,	,,	24/30	6·00
4	,,	,,	23/30	,,	,,	25/30	5·40
5	,,	,,	12/30	,,	,,	25/30	7·85
6	,,	,,	14/30	,,	,,	17/30	5·51
7	,,	,,	12/30	,,	,,	23/30	7·22
8	,,	,,	21/30	,,	,,	26/30	6·15
						Mean	6·08
			Sample C.				
1	1/1000	0·5 cc.	21/30	1/250	0·5 cc.	15/30	4·11
2	,,	,,	17/30	,,	,,	17/30	5·00
3	,,	,,	22/30	,,	,,	18/30	4·46
4	,,	,,	22/30	,,	,,	20/30	4·65
5	,,	,,	20/30	,,	,,	19/30	4·83
6	,,	,,	18/30	,,	,,	17/30	4·83
7	,,	,,	24/30	,,	,,	22/30	4·61
						Mean	4·64
			Sample D.				
1	1/1000	0·5 cc.	13/30	1/250	0·5 cc.	6/30	3·69
2	,,	,,	10/27	,,	,,	8/27	4·61
3	,,	,,	16/30	,,	,,	8/30	3·74
4	,,	,,	10/30	1/200	,,	11/30	4·12
5	,,	,,	22/30	,,	,,	16/30	3·69
						Mean	3·97

Estimates by H. P. Marks.

For estimating the potency of the various samples of crystalline insulin, the rabbit cross-over method was used, on the lines laid down in the original communications on this test [Marks, 1925, 1926], but with the following modifications, suggested by later experience.

(1) *Weight of rabbits and dosage of insulin.* Culhane [1928] states that the test gives more consistent results when the two groups of rabbits are so chosen that, for each animal in one group, there is a corresponding animal of approximately the same weight in the other group. This procedure was therefore adopted, and it has the added advantage that the same dose of insulin may be given to all the rabbits regardless of body-weight, without disturbing the balance of the two groups.

With one or two exceptions, rabbits weighing between 2 and 3 kg. were used, and the dose of standard insulin throughout was 1 unit per rabbit, injected subcutaneously.

The standard solution was diluted ten times for use, so that the volume injected was in every case 0·5 cc. Either 10 or 12 rabbits were used for a test.

The danger of choosing a dose falling on the upper, flat portion of the dosage-response curve has already been pointed out [Marks, 1926]. In order to avoid obtaining a false match between sample and standard, the dose of sample to be compared with 1 unit of the standard was accordingly varied from one test to another, and, in all cases, this was reflected as a change in relative response.

(2) *Treatment of blood samples.* The simplified method of dealing with the samples of blood already suggested in the communications cited above was adopted. It consists in combining the five hourly blood samples taken from the same rabbit after insulin. The blood-sugar value determined on this composite sample, when subtracted from the initial value, then gives the average fall in blood-sugar during the five hours.

According to Sahyun and Blatherwick [1928], if a rabbit is given a certain dose of insulin after a fasting period of 24 hours, and the fasting continued and the same dose of insulin given again after a further 24 hours, the hypoglycaemic response to each dose is approximately the same. This observation seemed to offer such an attractive possibility of shortening the time required for the cross-test that the matter was immediately investigated, and it was found that to the same dose of insulin, repeated on the second day, the response was slightly greater than on the first day. Although there were occasional exceptions on the part of individual rabbits the results were certainly no less consistent than if the rabbits had been fed after the first test, and prepared again for a second test two or three days later.

In all tests except the first test on A, and the final test on D, the following procedure was therefore adopted.

Food was withdrawn from the rabbits in the afternoon, and on the following day the first part of the cross-test was performed as usual. Instead of feeding the rabbits after the test, fasting was continued and the second part of the test was carried through on the next day, after which the rabbits were fed. Rabbits used in this way regularly once a week (*i.e.* one complete test per week) suffered no ill effects, and increased in weight at the usual rate.

The results of the tests are collected in Table II.

Table II.

Sample	Dose of solution in cc. per rabbit	Estimated units in this dose	Units per mg. of crystals
A	0·167	0·88	26·0
	0·2	1·00	24·4
			Average 25·2
B	0·2	1·07	22·6
	0·187	0·92	20·8
			Average 21·7
C	0·2	0·90	22·0
	0·22	0·96	21·4
			Average 21·7
D	0·2	0·89	27·4
	0·225	0·99	26·9
	0·25	1·01	24·6
			Average 26·3

The estimated activity of the dose was in all cases calculated by reference to the dosage-response curve already published.

Estimates by K. Culhane[1].

The determination of the potency of the four solutions was carried out by the cross-over method of Marks [1926] with no modifications except that certain precautions were taken with regard to the selection of suitable animals and the distribution of their weights [Culhane, 1928].

(1) *Choice of rabbits and dosage of insulin.*

The rabbits were selected from young animals which had been used previously for testing a few of our purest samples and found to give consistent results. These were divided into groups of approximately equal weights, the weights of all falling between 1600 and 2400 g. The standard was diluted so as to contain 2 units per cc. and the samples were also diluted to a volume expected to contain the same amount per cc., all doses, both of the sample and of the standard being adjusted to the weight of individual rabbits: the dose of the standard was always 0·5 unit per kg. of body weight.

In almost all of the tests 20 rabbits were used, but on rare occasions one or two were excluded from the result. This was only done when the rabbit

[1] It is a pleasure to acknowledge my indebtedness to Dr S. W. F. Underhill, Director of the Insulin A.B. Physiological Laboratories, for his valuable help and criticism during this investigation.

showed signs of illness during the course of the test or went into convulsions on one of the doses or gave a percentage reduction greater than 50 %, since such an animal is considered too sensitive for quantitative work.

(2) *Preliminary starvation period.*

All animals were starved for 22 hours preceding the test and fed immediately after the fifth hour after the injection. The second half of the test was carried out three or four days later after the same period of starvation.

(3) *Treatment of blood samples.*

To enable us to use so large a number of animals in one test the five hourly blood samples for each rabbit after injection were mixed and a practical average thus determined.

(4) *Calculation of results.*

Experience has shown that results which differ greatly from 100 % are often inaccurate, and, whilst there appears to be a general tendency for the error to cause them to approach nearer to 100 %, we have found it more useful to disregard such tests, except as an indication of a more suitable dose for the next, than to include their results in an average after applying the correction suggested by Marks [1925]. Hence, the result of the final test gives our conclusion for the potency of any solution. In one case only have we departed from this procedure, namely that of the last sample, of which only a small quantity was available; as all the results come within 86 and 89 % there seemed no reason for regarding one test as more accurate than another and the three have therefore been averaged, allowance being made for the number of rabbits used in each.

(5) *Results of the tests.*

The tests are summarised in Table III.

Table III.

Sample	Dose of solution in cc. per kg.	Result as percentage of standard	Units per cc.	Units per mg. of crystals
A	(1) 0·083	79	4·7	
	(2) 0·106	87	4·1	20·1
	(3) 0·125	102	4·1	
B	(1) 0·125	107	4·3	18·2
C	(1) 0·119	104	4·4	21·6
D	(1) 0·119	89	3·7	
	(2) 0·135	86	3·2	20·7
	(3) 0·135	89	3·3	

SUMMARY OF RABBIT TEST RESULTS.

Since not more than three rabbit tests were performed by either worker on any one sample, it is impossible to form any opinion as to the comparative degree of accuracy of the two sets of figures. It is readily apparent, however, that Culhane's figures show less variation in activity between the different samples than do those of Marks. If all the tests carried out by each worker

respectively are considered on the assumption that all the samples have the same real activity, we find that the average of all Culhane's results (excluding test A 1, as being below 80 %) is 20·3 with a standard deviation of 0·5 for the mean, while the corresponding figures for Marks's tests are 24·0 and 0·75 respectively. Put in another way we may say that the probability is 0·95 that the true mean value lies between 19·3 and 21·3, according to Culhane's figures, or between 22·5 and 25·5 according to Marks's figures. Taken as a whole, all the rabbit tests give an average figure of 22·3 with a standard deviation of 0·7 for the mean, *i.e.* the probability is 0·95 that the true value lies between 20·9 and 23·7.

Conclusions and discussion of combined results.

If we review the results obtained by the different investigators, as presented in the preceding sections, it becomes clear at once that there is no wide divergence between the values obtained by any one of us for the four different samples. The largest such difference is that between B or C and D obtained by Marks, but as this is not supported by the other investigators, no significance can be attached to it.

A comparison of Culhane's results with those obtained by Scott and Trevan would suggest that the rabbit test gives a consistently lower value for the crystals than the mouse test, but against this must be set the relatively close agreement between Marks's average of 23·7 for all four samples, and the average of 24·5 for all the mouse tests. It would indeed not be surprising if the rabbit test and mouse test should give somewhat different results when comparing insulin preparations of different degrees of purity, since the physiological reaction forming the basis of measurement is different in the two cases. Even if we assume that the crystals represent pure insulin, we must suppose, from our own data, that the standard preparation contains approximately two-thirds of its weight of other substances, of which some may not be physiologically inert. If the resultant effect of these should be to inhibit, even slightly, the convulsant action on mice, while disturbing less the average hypoglycaemic effect produced in a rabbit over a period of hours, the result would be to show a higher unit value for the crystals in the mouse test than in the rabbit test. Again, differences in the rate of absorption of the injected insulin, if such occurred, might account for the discrepancies observed. We can only say that the course of the hypoglycaemia, which was studied in some of the rabbit tests, gives no clear indication of any difference in this respect between the crystalline insulin and the International Standard. Realising these inevitable uncertainties of the comparative methods of biological measurement, we consider that the fairest procedure is to average the results for the whole series. In Table IV, therefore, we have averaged the results of each investigator for all four samples, and of all four investigators for each sample.

Table IV.

	A	B	C	D	Average
Marks	25·2	21·7	21·7	26·3	23·7
Scott	24·9	25·0	25·3	24·2	24·8
Trevan	24·2	25·8	22·8	24·4	24·3
Culhane	20·1	18·2	21·6	20·7	20·2
Average	23·6	22·7	22·9	23·9	

Looking at the results as a whole, we think that the two questions which we propounded at the outset may fairly be answered as follows.

1. Different batches of the crystals, prepared by different methods, have no differences of activity which the available methods of assay can detect.

2. The activity shown by all the four samples assayed in comparison with the International Standard, and based on the average of the values obtained by the four investigators for each sample, may be assessed at 23·3, with a standard deviation for the mean of $\pm$ 0·6.

This latter result is in close conformity with the values of 23 and 26 units obtained by Freudenberg and Dirscherl [1928] for the particular sample of the crystals which they tested. We can state with some confidence that a sample of such crystals as we have tested, if adopted in the future as a stable standard of reference, might be assumed to possess three times the activity of the present standard, *i.e.* 24 units per mg., without any significant disturbance of the biological unit, as defined in terms of the current International Standard.

The uniform activity of the four batches of crystals seems to us to indicate that their substance has a closer relation to the specific insulin activity than that of an inert adsorbent of an intensely active contaminant. On the other hand, the activity of the crystals is little, if at all, different from that of the most active amorphous insulin, obtainable by pushing to the limit the fractionations used in ordinary manufacture. We are familiar with preparations, commercially available in the ordinary way, in which the unit value per mg. is always in the neighbourhood of 20 and sometimes as high as 22 units per mg. The production of the crystals from such material would accordingly not entail the separation of a highly active principle from a crude mixture, but rather the creation of conditions allowing the crystallisation of a substance already almost pure. It would be analogous rather to the crystallisation of serum-albumin, for example, than to the isolation of such a hormone as adrenaline.

In conclusion we wish to express our indebtedness to Dr H. H. Dale, F.R.S., for his continued interest in this work, and for placing the facilities of his laboratory at the disposal of one of us (D.A.S.) for the animal experiments. We wish especially to thank him for his cooperation in arranging the physiological assay, which could not have been so extensive without his help.

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XLVII. STUDIES IN CARBOHYDRATE METABOLISM.

IV. ACTION OF HYDROXYMETHYLGLYOXAL UPON NORMAL AND HYPOGLYCAEMIC ANIMALS.

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Three compounds containing three carbon atoms have frequently been considered as possible intermediaries in carbohydrate metabolism: glyceraldehyde, dihydroxyacetone, and methylglyoxal. If the formula of hydroxymethylglyoxal, $CH_2OH.CO.CHO$, is compared with those of these three compounds it is seen that it bears a close relationship to all of them, and may, in fact, be regarded as an oxidation product of any of them. It was therefore considered of some interest to ascertain the action of this compound on the animal organism. This appeared the more desirable since evidence has been advanced by Hynd [1927] and Herring and Hynd [1928] which, it is claimed, supports the view that glucosone, $CH_2OH.(CH.OH)_3.CO.CHO$, is amongst the products formed in the animal body by the oxidation of dextrose under the action of insulin. In particular, they have found that the administration of glucosone to various animals causes the appearance of symptoms similar to those associated with insulin hypoglycaemia in the same species. The suggestion is that glucosone is the compound directly responsible for the appearance of these symptoms. Hydroxymethylglyoxal may be regarded as the simplest compound analogous to glucosone, bearing in fact the same relation to the triose glyceraldehyde that glucosone bears to dextrose. It was therefore of interest to determine whether hydroxymethylglyoxal causes the appearance of toxic symptoms similar to those produced by glucosone.

It may be noted that White and Willaman [1928] have found that hydroxymethylglyoxal is readily fermented by *Fusarium lini*, the only other compounds showing an equal ease of degradation by this mould being dihydroxyacetone and the fermentable hexoses.

Experimental.

The hydroxymethylglyoxal used in the following experiments was prepared by the method of Evans and Waring [1926], with one slight modification. These authors obtained good yields of the compound by oxidising dihydroxyacetone with copper acetate, allowing the mixture to stand for 10 days at ordinary laboratory temperature. We have been unable to obtain a crystalline product under these conditions, but found that the syrup produced rapidly crystallised if the oxidation had been allowed to continue for 30 days at room temperature or for 25 days at 25°. This difference is probably due to the fact that the laboratory temperature was considerably below that which prevails in American laboratories. With this modification we found that the reaction proceeds and the product crystallises just as described by Evans and Waring. It may be mentioned here that these authors found that when solid hydroxymethylglyoxal is dissolved in cold water it exists in a dimeric form, but when heated for 5 minutes at 100° it changes into the monomeric state.

Effect of hydroxymethylglyoxal on normal mice.

Experiments were first of all carried out to ascertain the effect of hydroxymethylglyoxal on normal mice, first with a freshly prepared solution of hydroxymethylglyoxal which had not been heated. The results are summarised in the following table.

Table I. *Effect of injections of unheated hydroxymethylglyoxal solution on mice.*

No. of exp.	Colour of mouse	Vol. of solution injected cc.	Concn. of hydroxy-methyl-glyoxal %	Wt. of hydroxy-methyl-glyoxal g.	Remarks
1	Albino	0·4	15	0·06	Sprawling in 1 minute then lying on side comatose. Breathing shallow, then gasping. Death in 3½ minutes
2	Albino	0·2	15	0·03	Arrest of respirations, convulsions and death in 2 minutes
3	Albino	0·4	7·5	0·03	Convulsions and death in 3 minutes
4	Black and white	0·3	7·5	0·0225	Convulsions, coma and death in 3 minutes
5	Black and white	0·2	7·5	0·015	Drowsiness, tail erected, paddling movements, coma, followed by recovery
6	Black	0·2	7·5	0·015	Erection of tail, convulsions, running movements, coma and death in 3 minutes
7	Black and white	0·2	7·5	0·015	Drowsiness, sprawling, paddling movements. Recovery in 2 hours
8	Albino	0·2	7·5	0·015	Convulsions, coma and death in 4 minutes
9	Black	0·2	7·5	0·015	Convulsions, coma and death in 4 minutes
10	Black	0·2	7·5	0·015	Drowsiness and sprawling, no convulsions. Recovery
11	Black	0·2	7·5	0·015	Drowsiness and sprawling, no convulsions. Recovery
12	Black and white	0·4	0·75	0·003	No symptoms

It will be seen that the lethal dose for a mouse is approximately 0·015 g. In those experiments in which death did not occur rapidly the animals exhibited a train of symptoms resembling those resulting from insulin hypoglycaemia. Thus in mouse No. 5, after a preliminary period during which the respirations were accelerated, the animal became drowsy for a time, then later the respirations became shallow and irregular, the tail was erected and the animal showed running movements. It then became comatose and lay on its side, respiration being barely perceptible, and in fact it was to all appearances moribund. After 20 minutes however, it showed signs of recovery, the respirations increased and the animal sat up unsteadily, the legs sprawling and the tail showing the peculiar stiffening commonly noted in the mouse during insulin hypoglycaemia. It remained in a languid condition for 3 to 4 hours and ultimately recovered completely.

The second series of experiments was carried out with a solution of hydroxymethylglyoxal which had previously been heated to 100° for 10 minutes. The results are summarised in Table II.

Table II. *Effect on mice of injections of hydroxymethylglyoxal solution, heated to* 100° *for* 10 *minutes.*

No. of exp.	Colour of mouse	Vol. of solution injected cc.	Concn. of hydroxy-methyl-glyoxal %	Wt. of hydroxy-methyl-glyoxal g.	Remarks
1	Brown	0·2	7·5	0·015	No symptoms
2	Albino	0·4	7·5	0·03	No symptoms
3	Black	0·2	15	0·03	Temporary excitement. No other symptoms
4	Albino	0·2	15	0·03	Transient muscular weakness; sprawling. No other symptoms
5	Albino	0·2	15	0·03	Became inactive for a short time. No other symptoms
6	Albino	0·4	15	0·06	After 8 minutes weakness of extremities. Moved about with difficulty. No convulsions. No coma. Breathing gradually became shallower. Death in 3 hours

It will be seen that the lethal dose of monomeric hydroxymethylglyoxal is 0·06 g., which is about four times the lethal dose of the unheated material. It appears, therefore, that the depolymerisation considerably reduces the toxicity of hydroxymethylglyoxal. Further, the depolymerised substance when administered in sublethal doses does not appear to cause the appearance of symptoms similar to those of insulin hypoglycaemia.

In the preparation of hydroxymethylglyoxal, it appears very difficult to eliminate the last traces of hydrogen sulphide and colloidal sulphur. The sulphur partially separates when the solution is heated to 100°. It seems, however, that hydrogen sulphide plays no part in the production of the toxic symptoms, since no symptoms were produced in control experiments

in which much larger quantities of hydrogen sulphide were employed than were contained in the unheated solution of hydroxymethylglyoxal. Further, the very small precipitate formed by heating the solution had no toxic effects.

Effect of hydroxymethylglyoxal on hypoglycaemic mice.

Ten mice were brought into a hypoglycaemic condition as the result of the administration of 0·2 cc. of standard insulin solution (20 units per cc.), diluted 1 in 25. To five of these mice sublethal doses of an unheated solution of hydroxymethylglyoxal were administered, and to the other five sublethal doses of a solution of hydroxymethylglyoxal previously heated to 100° for 15 minutes. In no case did recovery take place, but the animals recovered promptly after the administration of glucose. Hydroxymethylglyoxal is evidently without effect on insulin hypoglycaemia. It may be added that dextrose is without effect on the toxic symptoms produced by hydroxymethylglyoxal.

Effect of hydroxymethylglyoxal on rabbits.

The above results have all been confirmed by experiments on rabbits. These animals do not recover from hypoglycaemia as the result of the administration of hydroxymethylglyoxal, but rather are made worse, and death frequently follows in 10 or 15 minutes when the unheated material is used. This fatal result was observed even with doses which, when administered subcutaneously to normal animals, caused no obvious symptoms. In this respect the result is analogous to that previously observed with methylglyoxal [Kermack, Lambie and Slater, 1927]. As much as 9 cc. of a 25 % solution of hydroxymethylglyoxal, administered subcutaneously, produced no effect even when the solution had not been heated. When unheated hydroxymethylglyoxal was administered intravenously, 0·25 g. was sufficient to cause asphyxial convulsions and cessation of respiration following a momentary stimulation of the breathing. After generalised convulsions the animal lay on its side, showing running movements and intermittent gasping respirations. Artificial respiration was performed and the animal gradually recovered. On the other hand intravenous administration of 1·25 g. of a solution of hydroxymethylglyoxal, which had previously been heated to 100° for 10 minutes, produced no toxic symptoms. In relation to the results of Hynd, which have been mentioned above, an experiment may be reported here in which enormous doses of insulin (up to 200 units), were administered to rabbits and at the same time very large quantities of dextrose, to prevent reduction in blood-sugar. As expected, no hypoglycaemic symptoms occurred. If insulin produced hypoglycaemic convulsions as the result of the conversion of dextrose into glucosone it appears probable that such symptoms would have been observed in these experiments.

Discussion.

The above experiments demonstrate the highly toxic nature of hydroxymethylglyoxal in its dimeric form. Herring and Hynd [1928] state that 2·4 mg. of glucosone per g. body weight is sublethal for a mouse. In the case of dimeric hydroxymethylglyoxal subcutaneous administration of 0·75 mg. per g. body weight is sufficient to cause death. The symptoms produced by dimeric hydroxymethylglyoxal are similar to those described by Hynd and Herring as being produced by glucosone. On the other hand, 3 mg. of the monomeric form of hydroxymethylglyoxal per g. body weight were necessary to cause death in the mouse and the animal did not exhibit the characteristic train of symptoms above noted. It is surprising to find that the monomeric form, which gives the Schiff's reaction more readily than the dimeric form, and of which the aldehyde groups are therefore in a more active condition, is the less toxic. As pointed out by Olmsted and Logan [1923], the symptoms of insulin hypoglycaemia resemble those produced by asphyxia in the rabbit and cat. It would appear that many substances which interfere with the normal oxidative processes in cells (*e.g.* such as cyanide poisoning, cf. Hess [1921], carbon monoxide poisoning, etc.), tend in the lower animals to produce a train of symptoms which have many features in common, especially within the same species. Among these symptoms are muscular weakness (as shown by sprawling of the limbs or drooping of the head), head retraction, weakness, drowsiness, convulsive seizures with paddling and running movements, and ultimately coma. In the absence of other evidence, it would appear particularly hazardous to conclude that glucosone plays a part in the production of the symptoms of insulin hypoglycaemia because of the resemblance of these symptoms to those produced by insulin. The above experiments show that almost identical effects are produced even more readily by hydroxymethylglyoxal. It is also difficult to explain the non-appearance of such symptoms after the administration of enormous doses of insulin, provided that sufficient dextrose is given to prevent a fall in blood-sugar below the normal. Reference may also be made to the fact that in cases of diabetic coma large quantities of insulin are often given without harmful effect as long as the blood-sugar is not allowed to fall unduly. Even Herring and Hynd [1928] find it necessary to suggest that the sensitiveness of the animal to glucosone depends on the blood-sugar being abnormally low; and so in the absence of independent evidence as to the existence of glucosone in the animal body it does not seem to simplify matters to assume the development of a specific poison to explain the symptoms. It must also be remembered that the symptoms of glucosone or hydroxymethylglyoxal poisoning are not relieved by the administration of dextrose.

In its inability to relieve the symptoms of insulin hypoglycaemia, hydroxymethylglyoxal resembles the majority of compounds containing three carbon atoms of which, so far, only one, namely dihydroxyacetone, has been found to possess the power of bringing about rapid recovery.

SUMMARY.

1. Hydroxymethylglyoxal in its dimeric form is highly toxic to mice and rabbits and in sublethal doses produces symptoms similar to those of insulin hypoglycaemia.

2. In its monomeric form the toxicity is reduced by 75 % and the same train of symptoms is not produced.

3. Hydroxymethylglyoxal, either in the dimeric or monomeric form, is unable to cause recovery from insulin hypoglycaemia.

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XLVIII. STUDIES IN CARBOHYDRATE METABOLISM.

V. EFFECT OF ADMINISTRATION OF DEXTROSE AND OF DIHYDROXYACETONE UPON THE GLYCOGEN CONTENT OF MUSCLE IN DEPANCREATISED CATS.

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Introduction.

It has been shown by Burn and Dale [1924] and by Lambie [1926] that administration of insulin to decerebrated and eviscerated cats results in a more rapid disappearance of dextrose from the blood stream than when no insulin is given. It has further been shown by Best, Hoet and Marks [1926] and by Best, Dale, Hoet and Marks [1926] that a large proportion of the sugar which thus disappears is laid down as glycogen in the muscles.

Much discussion has centred around the question as to whether the essential action of insulin is to convert dextrose into some intermediary substance such as dihydroxyacetone. It therefore seemed desirable to carry out experiments with a view to ascertaining whether dihydroxyacetone causes the deposition of glycogen more readily than does dextrose. Mostowski [1911] showed that the administration of dihydroxyacetone to chickens results in the deposition of glycogen in the liver, whilst in more recent years Isaac and Adler [1924] have claimed that dihydroxyacetone, when injected intraperitoneally into mice and rats, effects more rapid deposition of glycogen in the liver than does dextrose. Cori and Cori [1928] have studied the distribution of glycogen in the livers and muscles of rats fed on these sugars, and found that at the end of 4 hours the rats fed on dextrose deposited 18 % of the sugar absorbed as glycogen in the liver and 25 % as muscle-glycogen, whereas in the case of dihydroxyacetone 21 % was laid down as liver-glycogen and only 15 % as muscle-glycogen. When insulin was administered the distribution of glycogen as between liver and muscle was 6 % and 36 % for dextrose and 2 % and 33 % for dihydroxyacetone, respectively. In both cases dihydroxyacetone failed

to lay down glycogen in the muscles as readily as dextrose but in the case of the liver the percentages were approximately the same. As Cori and Cori point out, these figures cannot be taken as representing the ease of formation of glycogen, since the fraction deposited in the muscles would, other things being equal, be decreased either by increased oxidation or by increased deposition in the liver. No conclusion can therefore be drawn from these experiments as to the relative ease with which the two sugars form muscle-glycogen.

Campbell and Markowitz [1927, 1, 2] have brought forward evidence which seems to prove that in the hepatectomised animal the administration of dihydroxyacetone is unable to cause recovery from the symptoms of hypoglycaemia and that the triose is quite unattacked by the organism under these conditions. These results suggest that dihydroxyacetone is converted in the liver either into dextrose or into some active form of the triose. It is known that dihydroxyacetone can, under certain conditions, be converted by the liver into dextrose, but, whilst this would account for certain resemblances in the physiological behaviour of the two sugars, the differences which they exhibit could not be satisfactorily explained on this hypothesis alone. It therefore seemed possible that experiments on animals in which the liver was intact might afford evidence that an active substance was formed from dihydroxyacetone. The following experiments were therefore carried out upon decerebrated and depancreatised cats in which the liver remained intact.

Experimental.

Cats were employed in all experiments. After brief etherisation the brain was destroyed with a probe through a trephine opening in the skull, and artificial respiration was carried out by means of a cannula inserted into the trachea. Cannulae were also placed in the left jugular vein and the left carotid artery. After withdrawing a sample of blood for analysis (Blood 1 in table), the abdomen was opened and the pancreas was removed. In one experiment only (No. 19 in table) was the animal eviscerated and the liver and kidneys tied off, thus excluding them from the general circulation. After pancreatectomy or evisceration, as the case may be, a second sample of blood (Blood 2 in table) was taken and then transfusion of the sugar under examination was begun. The sugar (dextrose or dihydroxyacetone) was dissolved in saline in the requisite concentration and, by means of the apparatus previously described [Lambie, 1926], was run in at a constant rate through the cannula in the jugular vein. Immediately after the perfusion had begun a ligature was placed round the right femoral artery and the muscles of the right lower limb were removed, cut into small pieces with scissors, weighed *en masse*, and then dropped into boiling 60 % potassium hydroxide solution. After the transfusion had continued for a given time, a third sample of blood (Blood 3 in table) was withdrawn and the left hind limb was removed and added to potassium hydroxide solution after cutting and weighing. The

glycogen content of the muscles was then determined by means of the following method.

Estimation of glycogen in muscle.

The weighed muscle (about 100 g.) was dropped into a conical flask containing hot aqueous 60 % potassium hydroxide (100 cc.) as described above and the mixture heated in a boiling water-bath for about 6 hours when the solid material had been practically completely decomposed and a reddish brown solution remained. On cooling, this solution was poured into a standard 250 cc. flask and diluted with water to the mark. After thorough mixing 5 cc. of the solution were pipetted into a 25 cc. centrifuge tube. Absolute alcohol (20 cc.) was then added and the mixture allowed to stand for 20 hours when the glycogen was completely precipitated. The precipitated glycogen was centrifuged off and washed twice with 66 % alcohol, then with absolute alcohol and finally with ether. It was then dissolved in 2 % hydrochloric acid (10 cc.) and heated on the boiling water-bath for 4 hours. The acid solution was washed into a 25 cc. standard flask and diluted to the mark. The glucose present in a convenient volume of the solution was then estimated by the Hagedorn-Jensen method [1923] and the percentage of muscle-glycogen calculated from this result.

In certain experiments curare was employed in order to paralyse the motor nerve endings of the muscle and eliminate any muscular twitching which might alter the glycogen content of the muscles. A solution, in saline, of the residue formed by evaporation to dryness of an alcoholic extract of the bark of *Strychnos toxifera*, 1 cc. of which had in control experiments been found to paralyse completely the motor nerve endings of the muscles of the lower limb of the cat, was injected in the requisite dose immediately after decerebration had been performed and artificial respiration begun.

As it was advisable to avoid as far as possible stimulation of the pancreas, ergotamine tartrate (5 mg.) was injected 2 hours before the beginning of the experiment with a view to paralysing the terminations of the splanchnic nerves in the liver and thus limiting the initial hyperglycaemia occasioned by the destruction of the brain and the ether anaesthesia, until the pancreas could be removed. It was found, however, that the ergotamine, by paralysing the sympathetic endings in the heart, often caused excessive slowing of that organ and the resulting depression of the circulation was frequently so great that circulatory failure followed the administration of the anaesthetic. When this threatened, adrenaline, 0·2 cc. of 1 in 1000 solution, was injected intravenously in order to save the animal. The results are summarised in Table I.

The total reducing power of the blood, as given in the table, in terms of glucose, was determined by the method of Hagedorn and Jensen [1923], whilst the method of Campbell [1926] was used for the determination of dihydroxyacetone. It was found by the Hagedorn and Jensen method that 1 g. of dihydroxyacetone is equivalent to 1·48 g. of glucose by this method.

Discussion.

The single experiment in which the liver was excluded from the circulation and the animal perfused with dihydroxyacetone showed a slight fall in muscle-glycogen in spite of the fact that the blood at the end of the experiment contained 0·1 % of dihydroxyacetone. This seems to indicate, in agreement

Table I.

No. of exp.	Wt. of cat g.	Sugar perfused	Concn. of sugar in perfusion fluid %	Rate of perfusion per hour g.	Time of perfusion mins.	Total wt. of sugar perfused g.	Blood-sugar (total reducing power) mg. per 100 cc. 1	2	3	Glycogen in muscle mg. per 100 g. Before	After	Difference	Remarks
1	2600	Dextrose	40·6	2·02	90	3·04	276	369	1560	234	298	+ 64	Pancreas removed.
2	—	,,	40·0	2·0	130	4·30	222	276	318	253	201	− 52	Liver intact
3	2300	,,	34·5	1·72	117	3·44	—	—	—	476	170	−306	Curare. Pancreas removed. Liver intact
4	—	,,	30·0	1·5	175	4·50	84	106	588	359	238	−121	Ergotamine. Pancreas removed. Liver intact
5	—	,,	30·0	1·5	100	2·50	282	438	—	307	455	+148	
6	—	,,	30·0	1·5	135	3·37	254	326	580	574	562	− 12	Ergotamine. Adrenaline. Pancreas removed. Liver intact
7	—	,,	60·0	6·25	106	11·04	144	198	2220	279	206	− 73	
8	—	,,	30·0	1·5	130	3·25	—	104	456	658	687	+ 29	
9	2300	Dihydroxyacetone	30·0	1·5	120	3·00	258	326	870	402	407	+ 5	
10	3200	,,	50·0	2·5	70	2·91	—	318	1560 (0·42 % Di.)	194	242	+ 48	
11	2250	,,	50·0	2·5	90	3·75	140	158	1140 (0·1 % Di.)	592	421	−171	Pancreas removed. Liver intact
12	2270	,,	35·4	1·77	90	2·66	222	343	1020 (0·173 % Di.)	274	288	+ 14	
13	—	,,	40·0	2·0	80	2·66	258	404	1230	149	206	+ 57	
14	—	,,	40·0	2·0	110	3·66	154	220	1710	381	372	− 9	
15	2100	,,	31·5	1·58	120	3·15	—	—	—	274	223	− 51	Curare. Pancreas removed. Liver intact
16	2400	,,	37·4	1·87	90	2·80	—	—	—	499	577	+ 78	
17	—	,,	30·0	1·5	80	2·0	230	426	1008	559	743	+184	Ergotamine. Adrenaline. Pancreas removed. Liver intact
18	—	,,	60·0	6·25	90	9·37	272	282	3180	989	564	−425	
19	3450	,,	53·0	2·69	90	4·03	—	254	1140 (0·1 % Di.)	252	232	− 20	Complete evisceration. Liver tied off

The calculated Blood 3 glucose values in experiments Nos. 10, 11, 12 and 19 are 938, 992, 764 and 992 mg. per 100 cc. respectively.

with the conclusion of Campbell and Markowitz [1927, 1, 2], that glycogen is not produced readily from dihydroxyacetone in the absence of the liver, but further experiments are necessary to settle this point.

It will be noted that in certain experiments ergotamine or curare was administered to the animal. As explained above, this was done with the aim of preventing glycogenolysis in the liver or muscles respectively but there was no evidence that the curarised animals gave more constant results than the untreated ones, whilst the animals which had been treated with ergotamine frequently suffered from circulatory failure and usually died if not given adrenaline. Moreover the ergotamine was frequently ineffective in paralysing the splanchnics completely.

It is evident from the figures in the preceding table that no significant difference is observable under the above conditions in the power of dextrose and of dihydroxyacetone to cause deposition of glycogen in the muscles. In the eight experiments with dextrose the mean decrease in muscle-glycogen from the beginning to the end of the perfusion period was $0{\cdot}040 \pm 0{\cdot}051$ %, whilst when dihydroxyacetone was used the decrease was $0{\cdot}027 \pm 0{\cdot}054$ %. It is obvious, however, that in individual experiments a change in the glycogen is quite considerable and the question arises as to whether this can be correlated with any of the other factors which vary from experiment to experiment.

Inspection shows that with the exception of experiment No. 18, a marked fall in glycogen is usually associated with a lower initial blood-sugar, whilst a marked rise in glycogen is usually observed with a high initial blood-sugar. The figures (including Exp. 18) were therefore examined statistically, taking the 13 experiments in which the initial blood-sugar had been determined, in order to find out whether any correlation existed between the level of initial blood-sugar and the change in the muscle-glycogen. The value of the partial correlation coefficient between initial blood-sugar level and change in muscle-glycogen for a constant initial glycogen content was found to be $-0{\cdot}30 \pm 0{\cdot}15$. This correlation coefficient would have been considerably greater but for experiment No. 18. It may be noted that in this experiment an unusually high initial muscle-glycogen existed and the large fall in glycogen content may be related to this factor. Because of the comparatively small number of animals (13), the probable error of the correlation coefficient is high and too much emphasis must not be laid on the result, but the above experiments suggest that the level of the initial blood-sugar in such a perfusion probably plays a rôle in determining the alteration in glycogen. This may be due to the stimulation of the pancreas, in the short time that elapses before it is excised, so that it secretes insulin and in this way brings about the deposition of glycogen.

Whilst these experiments do not prove conclusively that dihydroxyacetone cannot cause deposition of glycogen in the muscles more readily than dextrose, they show that there is no striking tendency towards such glycogen deposition and to this extent are in accord with the observations of Cori and Cori which have been mentioned above. In some experiments, the amount of dihydroxyacetone administered was certainly large enough to effect a definite rise in muscle-glycogen provided the triose readily polymerised to form the polysaccharide. In experiment No. 18, in which 9 g. of dihydroxyacetone were administered, a fall of glycogen occurred of 0·425 %, which is by far the greatest fall observed in all experiments, and further, the four experiments in which a fall took place with dihydroxyacetone are those in which the animal received the largest amounts of the triose, and the animal which received the smallest amount of dihydroxyacetone showed the biggest rise. If the correlation coefficient between the amount of triose administered and the change in glycogen be calculated it is found to be $0{\cdot}95 \pm 0{\cdot}02$, which indicates a strong relation between fall in glycogen content and the amount of triose given. As mentioned above, too much emphasis cannot be laid upon the exact figure, because of the comparatively small number of animals used. When the experiments in which dextrose was administered are examined in a similar way, the correlation coefficient is found to be $0{\cdot}56 \pm 0{\cdot}15$. This also indicates the important effect which a large amount of dextrose has in causing the muscle-glycogen to fall. We do not at present propose to discuss the various theories which may be advanced to explain this remarkable result. Since the amount of carbohydrate injected has this effect upon the

disappearance of glycogen from muscle, it is obvious that no conclusion can be drawn, from experiments of this type, regarding the comparative ease with which glycogen can be laid down in muscle.

Summary.

1. When dihydroxyacetone is administered intravenously to decerebrated cats from which the pancreas has been removed, the change in muscle-glycogen depends largely upon the amount of triose administered. With small quantities an increase tends to occur, with large quantities a decrease.

2. When dextrose is administered under similar conditions, results of an analogous type are obtained, although, in this case, the relation between the amount of glucose administered and the change in glycogen may not be so great.

3. The level of the initial blood-sugar, before pancreatectomy, appears to have some effect upon the change in muscle-glycogen, high initial blood-sugars tending to cause an increase. This is probably due to stimulation of insulin secretion by the pancreas.

4. From the experiments, no evidence could be obtained that muscle-glycogen was more readily formed from dihydroxyacetone than from dextrose in presence of the liver.

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XLIX. THE ANTICOAGULANT ACTION OF ANTITHROMBIN.

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The presence of a substance in blood, which inhibited coagulation, was noted by early workers on blood coagulation. Schmidt [1892] stated that this substance was present in all cells. To it the term anti-fibrin-ferment was applied by J. Mellanby [1908] and antithrombin by Howell [1910]: the former, who found that the anticoagulant action was abolished by heating to 70°, observed that complete disappearance of thrombin could not be effected by the addition of an excess of antiferment and therefore concluded that the union of antiferment with ferment (thrombin) followed the law of mass action. According to Howell [1912] antithrombin enters into combination with prothrombin and also with thrombokinase, but does not combine with thrombin [cf. Bordet, 1920].

The anticoagulant action of blood upon a mixture of fibrinogen (plasma) and thrombin is observable in the presence of sodium citrate. All the experiments about to be described were made with citrated solutions. Under these conditions of experiment thrombokinase does not convert prothrombin into thrombin: the possibility of the coagulation time being affected by any concomitant interaction of thrombokinase and prothrombin, occurring in the plasma employed, is thus avoided. The action of the anticoagulant must, therefore, be exerted either upon fibrinogen or upon thrombin and is presumably similar to the effect of simple dilution of one or other of the solutions of these two substances employed.

The action of serum in producing delay of coagulation in presence of sodium citrate, is illustrated by the following two experiments (quantities of liquid being given in cc.)[1]:

Table I.

Exp.	Plasma	Serum	0·85 % NaCl	Thrombin	Onset of coagulation
1	0·08	—	0·014	0·026	2·5 minutes
2	0·08	0·014	—	0·026	4·5 „

It will be observed that the coagulation time is nearly doubled by the addition of a relatively small amount of serum.

A similar result is obtained if plasma is employed instead of serum.

[1] As source of thrombin the venom of *Echis carinatus* [cf. Barratt, 1913] was used. I am indebted to Sir Charles Martin, F.R.S., Director of the Lister Institute, for kindly providing me with a supply of this venom.

The anticoagulant was, however, found to be present in plasma in amount too small for experimental work: in particular the effect upon fibril formation, which it was desired to observe, could not be ascertained, the changes noted being slight and indefinite.

An attempt was therefore made to determine if the anticoagulant substance could be extracted from plasma. Ultimately it was found that the complete disappearance of this substance, which occurs on heating plasma or serum to 70°, does not take place in presence of a large excess of distilled water. Making use of this circumstance some measure of success was obtained by the following method of extraction which was employed for Exps. 3 to 6, given below.

5 cc. of human plasma were added to 45 cc. of distilled water and the diluted plasma so obtained was poured into 450 cc. of distilled water at 100°. After being kept at about 90° for 5 minutes, the mixture was cooled to 70°, at which temperature it was allowed to evaporate. In the course of $2\frac{1}{2}$ hours its volume was reduced to 20 cc. This was put into a parchment thimble and allowed to dialyse into a litre of distilled water for 48 hours. The liquid contained in the parchment thimble was then evaporated to dryness. The residue was extracted with distilled water, in which it in part slowly dissolved, the insoluble portion being separated by centrifugalisation and the supernatant liquid again evaporated to dryness. The residue thus obtained was then extracted with distilled water, more insoluble material appearing. The process of extraction was repeated several times until at length no further insoluble precipitate was obtainable. In this way a small amount of soluble residue forming a brownish hyaline scale was obtained. For the separation of insoluble protein removal of salts by dialysis was found to be essential.

A portion of this soluble extract, dissolved in 0·85 % NaCl solution, was mixed with citrated plasma. To the mixture thrombin was added (Exp. 3), a control experiment, in which 0·85 % NaCl solution free from extract was used, being also made (Exp. 4). These experiments were repeated with a slightly larger amount of extract (Exps. 5 and 6). The figures in brackets in Table II represent the amount of plasma corresponding to the extract employed.

It will be seen that in each case the addition of extract caused delay in the time of appearance of fibrils.

Table II.

Exp.	Plasma cc.	Extract cc.	0·85 % NaCl cc.	Thrombin cc.	Time of appearance of fibrils mins.
3	0·05	—	0·06	0·01	20
4	0·05	0·06 (0·25)	—	0·01	56
5	0·05	—	0·06	0·01	21
6	0·05	0·06 (0·4)	—	0·01	120

In order to determine whether this delay is due to an action exerted upon fibrinogen or upon thrombin, the effect upon the fibrils of the coagulum was

noted and compared with that occurring (1) when the plasma employed is diluted, and (2) when the thrombin added is similarly diluted: in the former case, in which the concentration of fibrinogen is diminished, the fibrils become short and fine, scanty and barely visible; in the latter, in which the concentration of thrombin is lowered, they are also reduced in number, but exhibit increase in length and thickness [Barratt, 1921].

In Table III the condition of the fibrils in Exps. 3 to 6 is given.

Table III.

Exp.	Approximate number of fibrils in field	Length	Thickness
3	3000	15μ to 40μ	0·1μ to 0·2μ
4	1000	20μ to 50μ	0·1μ to 0·2μ
5	4000	30μ to 60μ	0·1μ to 0·2μ
6	1200	75μ to 170μ	0·2μ to 0·4μ

It will be seen that a decrease in number of fibrils occurs when extract is added, accompanied by an increase in length and thickness. The anticoagulant substance thus causes diminution in the amount of free thrombin present and is, therefore, an antithrombin in the sense of causing such decrease.

It may be observed that the only means at present available of deciding whether the action of antithrombin upon a mixture of fibrinogen and thrombin in presence of sodium citrate is exerted upon fibrinogen or upon thrombin appears to be by noting the accompanying change of type of fibril formation. In this way an unequivocal indication of the mode of action of the anticoagulant is obtained.

Summary.

(1) Experiments were made to determine the effect of antithrombin, obtained from human blood-plasma after removal of precipitable proteins, upon the formation of fibrils in the coagulation of fibrinogen.

(2) It was found that the anticoagulant action of antithrombin upon a mixture of fibrinogen (citrated plasma) and thrombin *in vitro* is exerted upon thrombin, which becomes diminished in amount: no evidence of any action upon fibrinogen was observed.

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L. ON CARRAGEEN (*CHRONDRUS CRISPUS*).

IV. THE HYDROLYSIS OF CARRAGEEN MUCILAGE.

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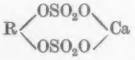
The chemical composition of carrageen has been the subject of investigation from time to time by various authors [*e.g.* Flückiger and Obermayer, 1868; Bente, 1875, 1876]. More detailed investigations were carried out by Haedicke, Bauer and Tollens [1887], who established the presence of fructose and estimated the galactose content at 28 %. Sebor [1900] confirmed the presence of galactose and fructose, found small quantities of pentosan and claimed to have found evidence of glucose, a question which will be referred to again later in the present paper.

All the above authors assumed the mucilage which is obtained by extracting the weed with water to consist of a single polysaccharide. Later, however, it was shown by Haas and Hill [1921] that the extract is in fact a mixture of two substances both of which are carbohydrate esters of sulphuric acid of the following type:



in which R stands for the carbohydrate radicle. These two ethereal sulphates, which were described as hot and cold extracts (H.E. and C.E. respectively), differed primarily in their solubilities and in the fact that the H.E. in 3 % solution forms a gel whereas the C.E. forms instead a viscous liquid. The present investigation is the outcome of an attempt to isolate the carbohydrate complex free from sulphate, but, as the result of a number of trials with various strengths of acid, it was found that the conditions which favour the separation from either H.E. or C.E. of the sulphate residue involve the complete breakdown of the carbohydrate complex.

It has been known that carrageen mucilage cannot be heated with dilute acids such as lemon juice or vinegar without sacrificing its gelatinising power. This observation seemed of some interest, and experiments were accordingly undertaken with the object of determining the strength of acid which would just destroy the gelatinising power of H.E. It was found that when a 2 % solution of H.E. was heated at 80° for 45 minutes with 0·15 *N* sulphuric acid the resulting liquid had acquired reducing properties, but, contrary to expectation, this was not due to the formation of free sugar but to the production of a non-dialysable product which proved to be still an ethereal sulphate.

In an actual experiment 250 cc. of 2 % H.E. were mixed with 250 cc. of 0·15 *N* sulphuric acid and heated on a water-bath for 45 minutes at 80°. The contents of the flask were then cooled and neutralised with calcium carbonate and, after filtration, evaporated to small bulk *in vacuo* and dialysed against distilled water for 2 days, then against running tap water for another 2 days, and finally once more against distilled water for a further 24 hours: the contents of the dialyser were then evaporated to dryness. The resulting product was designated the "residue."

The outer water from the first two days' dialysis was collected, evaporated to small bulk, filtered, heated to boiling, treated with a hot solution of baryta and left to stand overnight. After filtering, the excess baryta was precipitated with carbon dioxide, and the liquid heated to boiling, filtered and evaporated to dryness over a water-bath; this product was designated the "dialysate." As the residue was found to be slightly dialysable, the dialysate was further purified by being once again put into the dialyser, in this case only the first 12 hours' dialysate being collected and evaporated to dryness.

Examination of residue and dialysate.

Residue. This, consisting of light yellow horny scales and having an ash content of 19·75 %, was found to reduce Fehling's solution and to be optically active, specific rotation + 38·3°. It gave a positive Selivanoff reaction indicating the presence of fructose; the presence of pentose was also established by Bial's reagent and the production of furfural on heating with 12 % hydrochloric acid. On oxidation with nitric acid, crystals of mucic acid were obtained proving the presence of galactose. The solution gave no precipitate with barium chloride until after hydrolysis with hydrochloric acid, when a precipitate of barium sulphate was obtained which corresponded to a sulphate content of 26·72 %. An estimation of the sulphate contained in the ash obtained by incinerating a sample of the same material gave a value of 13·66 % calculated on the dry weight of the material. From these two figures the presence of an ethereal sulphate may be regarded as established [cf. Haas, 1921].

Dialysate. This was obtained as a brown, gummy material which it was not found possible to purify. It gave no precipitate with barium chloride until after hydrolysis with hydrochloric acid, from which it was inferred that it was also an ethereal sulphate. The substance gave the colour reactions for pentose and for fructose, but on oxidation with nitric acid yielded no mucic acid, showing the absence of galactose. It did not reduce Fehling's solution until after hydrolysis and was optically inactive.

The fact that the reducing properties were confined to the residue, which was unable to pass through a parchment dialyser, while the dialysable product had not reducing properties, showed that no free sugar had been produced as the result of the mild conditions of hydrolysis described above, since such sugars, if formed, would have passed out of the dialyser into the outer water.

From the description of the dialysate and residue given above it will be seen that they differ from each other and from the original H.E. The following table summarises the characters of the three substances:

	Dialysate	Residue	H.E.
Pentose (Bial's reagent)	+	+	+
Galactose (by mucic acid test)	−	+	+
Fructose (by Selivanoff)	+	+	+
Reduction of Fehling's solution	−	+	−
Optical activity	Inactive	Dextrorotatory	?*

* The optical activity of this material could not be satisfactorily determined owing to the fact that a sufficiently strong solution for the purpose is too opaque.

The occurrence of glucose in carrageen mucilage.

The presence of glucose in carrageen was originally suggested by Sebor [1900] on somewhat slender evidence; subsequently Müther and Tollens [1904] claimed to have established the presence of glucose on the basis of an analysis of the silver salt of a presumed saccharic acid. Since the silver content of saccharic acid is the same as that of mucic acid, a determination of the silver content furnishes no proof of the presence of glucose. It will be seen from the table given above that both dialysate and residue give the Selivanoff reaction for fructose before hydrolysis. When hydrolysed by heating on a water-bath for 3 hours with 5 % hydrochloric acid, however, the resulting solutions no longer gave the Selivanoff reaction, but on treatment with phenylhydrazine the residue alone gave a precipitate of glucosazone. Since the negative Selivanoff reaction showed the absence of fructose, the osazone could only have been formed from glucose.

In order to obtain further evidence in support of the presence of glucose, an attempt was made to prepare the acid potassium saccharate by oxidation with nitric acid; here a difficulty was encountered from the fact that, owing to the high calcium content of the material, the products of oxidation, namely mucic, oxalic and possibly saccharic acids, were obtained in the form of their sparingly soluble calcium salts. This circumstance makes it probable that saccharic acid, if present in small quantity, would escape detection, and in fact preliminary experiments failed to reveal any of this acid. To overcome this, recourse was had to the oxidation of a sample of the potassium salt of the H.E. For this purpose the mucilage was boiled on a water-bath with potassium carbonate, and subsequently filtered from the calcium carbonate formed. 2 g. of the resulting potassium salt were then oxidised with nitric acid, filtered from the crystals of mucic acid produced, and treated with anhydrous potassium carbonate and acetic acid in the usual manner. After the solution had stood for some days, perfectly typical crystals of acid potassium saccharate were obtained. This experiment, taken in conjunction with that in which glucosazone was obtained from the residue after the destruction of the fructose, establishes conclusively the presence of glucose in the mucilage of carrageen.

Alkaline hydrolysis.

A further attempt was made to isolate the carbohydrate residue by alkaline hydrolysis of C.E.[1] For this purpose various concentrations of aqueous sodium, potassium and barium hydroxides were successively tried both at the temperature of the water-bath and at higher temperatures in an autoclave; but, as in the case of acid hydrolysis, it was found that any attempt to split off the sulphate complex was accompanied by a breakdown of the carbohydrate with the formation in this instance of lactic acid and other products. Moreover, an examination of the products of alkaline hydrolysis revealed the fact that the quantitative liberation of the sulphate is a very slow process, as is shown by the following experiment. Portions of C.E. weighing 0·7 g. were heated with 40 cc. 3 % sodium hydroxide (free from sulphate) in an autoclave at 110° for varying lengths of time and the amount of free sulphate produced was estimated. The results obtained are presented below in tabular form. From the figures a curve was constructed which by extrapolation indicated that complete hydrolysis should have been effected in 54½ hours. Actual experiment showed, however, that out of a possible 30 % of sulphate only 20·5 % could be so obtained. This suggests that a portion of the sulphate is united more firmly than the rest, since it resists hydrolysis by 3 % sodium hydroxide at 110°.

Time heated hours	Sulphate obtained %	Time heated hours	Sulphate obtained %
1	10·42	9¼	18·28
2	12·16	11½	18·18
3	14·78	16¼	19·86
7	17·00		20·00

This conclusion is in agreement with the observations made upon acid hydrolysis where it was found that, while appreciable quantities of sulphate could be set free by a few minutes' boiling with dilute mineral acid, complete hydrolysis could only be effected by 6 hours' boiling with 4 *N* hydrochloric acid.

Action of alcoholic potassium hydroxide on H.E.

12 g. H.E. were dissolved in 160 cc. water and 320 cc. 3 % alcoholic potassium hydroxide added. The H.E. was precipitated, but in spite of this the whole was heated on a water-bath under a reflux condenser for 15 hours. Examination of the residue showed that three different substances had been produced, a portion soluble in cold water, another soluble in hot water only and a third which was insoluble in either hot or cold water. That none of these three was the sulphate-free carbohydrate complex was shown by the fact that they all contained a higher percentage of ash than the original material. The hot water-soluble portion was found to have a gelatinising

[1] In this case the C.E. was selected for experiment since it is an ethereal sulphate of the same type as H.E. but takes considerably less time to prepare.

power five times as great as that of the original substance. It was shown to be an ethereal sulphate and is probably the potassium salt of the original H.E.; previous attempts to prepare this salt by double decomposition from the original mucilage, which is a calcium salt, had resulted in a compound which also had a greater gelatinising power than the original but contained, like the original, a certain percentage of nitrogen, whereas the product here obtained was nitrogen-free. It was not further investigated.

SUMMARY.

(1) The loss of gelatinising power resulting from mild hydrolysis of carrageen is not accompanied by production of free sugars although reducing properties are developed.

(2) The solution resulting from mild hydrolysis of carrageen contains two ethereal sulphates, which can be separated by dialysis. The product which is retained by the dialyser has reducing properties; the substance which passes through has no reducing power.

(3) The presence of glucose has been established in carrageen mucilage.

(4) Alkali hydrolysis of a cold water extract is shown to result in complete breakdown of the carbohydrate complex, and it was found that a quantitative separation of the sulphate could not be effected by this means.

The authors acknowledge their indebtedness to Miss K. J. Bardsley, M.Sc., for a preliminary study of the behaviour of carrageen under varying conditions of acid hydrolysis.

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LI. THE ISOLATION OF A CARBOHYDRATE DERIVATIVE FROM SERUM-PROTEINS.

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Many investigators in the past have, at different times, called attention to the fact that preparations of proteins made by them persistently gave positive Molisch or orcinol tests. In no case, however, was any satisfactory evidence brought forward in support of the view that carbohydrate substances could be separated from proteins until Seemann [1898] obtained glucosamine from egg-albumin as the hydrochloride, after acid hydrolysis of the protein. Numerous other investigators have reported the isolation of osazones as a result of treating the acid hydrolysates of various proteins with phenylhydrazine hydrochloride in the customary manner. Thus Mörner [1893] obtained from serum-globulin an osazone melting at 170°–172°. Pavy [1895], Krawkoff [1896] and Jacewicsch [1897] also reported positive findings, whilst Langstein [1902] succeeded in isolating benzoylglucosamine after having benzoylated the mixture obtained by hydrolysing crystalline serum-albumin.

A very large amount of work has also been done upon the mucins, clearly proving that they are glucoproteins and establishing their conjugate character.

In the case of the simpler proteins—especially the albumins and globulins—agreement is by no means so general. Whilst researches such as those quoted above seem to indicate the probable presence of carbohydrate groups as integral constituents, the criticism is always possible that the starting material was not put through a sufficiently rigorous process of purification. Moreover, evidence of a more indirect nature has been sought by examining the power of hydrolysed proteins to reduce alkaline copper solutions and whilst many investigators have claimed positive findings in this direction the results of others have been directly opposed [Hofmann and Pregl, 1907]. Abderhalden, Bergell and Dörpinghaus [1904] deny the presence in serum-albumin of any carbohydrate capable of giving a positive Molisch reaction. Among such contrary findings it is difficult to draw any conclusion. The present author would suggest that the lack of unanimity is very probably due in large measure to the differing experimental conditions. Most previous workers have employed relatively concentrated acid to effect hydrolysis of the proteins. Under these conditions secondary reactions are liable to occur, involving amino-acids

and any carbohydrate that may be formed, and resulting in humin production. It is significant that in nearly all cases the carbohydrate recognised, or claimed to have been present, is glucosamine or an amino-sugar. Now glucosamine gives neither the Molisch nor the orcinol tests and therefore if these tests were originally positive the protein must also have contained some other carbohydrate substance.

Experimental.

In the present investigation very carefully purified proteins have been used and a method of hydrolysis employed which, whilst effective in breaking up the protein, had little or no action upon the carbohydrate substances formed.

Preparation of the serum-proteins.

Freshly drawn horse's blood was allowed to stand at 0° for 24 hours. The expressed serum (2 vols.) was separated off and centrifuged. It was then diluted with 5 vols. of water and 3 vols. of saturated ammonium sulphate were added. The small precipitate was filtered off and discarded. Ammonium sulphate was added to the remainder until half saturation. The precipitated globulin was separated by suction and the albumin precipitated by fully saturating with solid ammonium sulphate.

These two precipitates were purified by eight successive reprecipitations from large volumes of solution, the end fractions being discarded.

Each was then denaturated by addition of 5 vols. of alcohol and 30 cc./litre of a salt buffer mixture[1] (p_H 4·7) to its aqueous solution. After removal of the alcohol, the denaturated protein was washed by repeated extraction with large volumes of distilled water (5 litres to 150 g. protein) filtering by suction between each washing. Dehydration was finally accomplished by alcohol and absolute ether. The preparations so obtained were pure white in colour. Coloured filter papers were employed throughout so that any fine particles adhering to the precipitates could be easily seen and removed. Preparations of both albumin and globulin used in reasonable quantity were found to give a faint, but distinctly positive, Molisch reaction. The colour appeared to form somewhat slowly and was most intense after about half an hour's standing. Possibly hydrolysis in the presence of the acid was occurring, liberating the reactive agent.

Isolation of a carbohydrate derivative.

Hydrolysis was accomplished by boiling under a reflux condenser 150 g. of albumin or globulin with an equal weight of crystallised baryta and ten times its weight of water.

[1] Made up as follows.

Sol. A. 40 g. potassium sulphate, 202·5 g. sodium sulphate (cryst.), 360 cc. water heated to effect solution, then cooled. Sol. B. Normal acetic acid. Sol. C. Normal sodium acetate. Equa volumes of A, B and C mixed: p_H=4·7–4·8.

After 3½ hours the golden-coloured solution, which gave a strongly positive Molisch reaction, was cooled, filtered from a little insoluble material and the bulk of the barium removed by passing a stream of carbon dioxide through the solution at 60°.

After filtration, saturated lead acetate was added, and the slight precipitate filtered off and discarded, as it was invariably found to give no Molisch reaction. The carbohydrate was precipitated, however, by addition of ammonia which was continued as long as any precipitate formed. The filtrate gave no Molisch reaction, showing that separation was complete.

The lead-ammonia precipitate was filtered by suction, washed repeatedly on the filter with weak ammonia, finally with distilled water, and then ground into a cream with water. Upon acidifying with acetic acid most of the precipitate dissolved; the insoluble matter, which was usually pigmented, could be filtered off and discarded as it gave no Molisch reaction. Addition of excess of ammonia to this filtrate once more precipitated the carbohydrate. It was found by experience that much difficulty could be saved at later stages by repeating this washing and reprecipitation of the lead compound several times.

Finally it was suspended in water and decomposed by a stream of carbon dioxide at 60°. The point at which decomposition is complete can be easily ascertained with practice, for at this stage the suspended lead carbonate rapidly sinks to the bottom of the containing vessel.

The liquid was filtered. It was light yellow in colour, gave an intense Molisch reaction and pink biuret reaction, showing that it still contained protein substances. Most of the latter were removed by addition of mercuric chloride solution (saturated). It was necessary to add the reagent until further addition caused no turbidity and then to allow the mixture to stand in the laboratory for several days—10 or 12 preferably—as precipitation continued slowly for some time. The filtered liquid was now treated with hydrogen sulphide to remove excess of mercury and lead and after filtration a stream of air was drawn through to remove excess of gas. At this stage the solution was colourless or very pale yellow. It still gave a faint biuret reaction (pink) and an intense Molisch reaction but did not give any trace of turbidity with the following protein precipitants: uranium acetate, sodium tungstate, copper acetate. Phosphotungstic acid in 5 % sulphuric acid gave a white precipitate. This contained the carbohydrate but precipitation was not complete until a large excess of the reagent was present. The final stages of isolation took place as follows. The clear filtrate from the mercuric sulphide was concentrated under reduced pressure to a small volume. Four volumes of methyl alcohol were then added, followed by 5 vols. of absolute ether.

The carbohydrate material was precipitated as a sticky mass which adhered to the sides of the vessel and stirring rod. By leaving it in contact with absolute ether, its character changed completely, for on dehydration it broke up into a fine white powder, having an almost crystalline appearance.

After 5 or 6 reprecipitations by means of methyl alcohol and ether, it was

found that the nitrogen content remained constant at 4·4 to 4·9 % (in place of 5·5 % after the first precipitation). The material at this stage still gave a pink biuret reaction suggesting admixture with protein substances.

Much biuret-giving material was eliminated by addition of glacial acetic acid to the concentrated aqueous solutions and, after 2 or 3 precipitations by ether, the anhydrous material was found to have a constant nitrogen content of 4·1 %. The products isolated from albumin and globulin respectively were apparently identical. The yield from each protein was just under 2 % of pure material.

Microanalysis[1] yielded figures which, after correction for a slight amount of ash (about 0·2 %), agreed with the formula $C_{12}H_{23}O_{10}N$. A small but variable amount of chlorine was also present (about 3 %) and this has been allowed for in reckoning the percentages.

Carbohydrate from albumin.

Prep. I. Total N 3·99, 4·28, 3·94 %.
Prep. II. C 41·57, 42·89; H 7·26, 7·61; N 3·99, 4·14 %.
Calcd. for $C_{12}H_{23}O_{10}N$: C 42·21, H 6·79, N 4·10 %.

Carbohydrate from globulin.

Prep. I. Total N 3·98, 4·17 %.
Prep. II. C 41·73, 42·71; H 7·01, 6·75; N 4·19 %.
Calcd. for $C_{12}H_{23}O_{10}N$: C 42·21, H 6·79, N 4·10 %.

Properties of the carbohydrate derivative.

The substance isolated appears to be a nitrogen-containing polysaccharide. It is extremely hygroscopic, dissolves readily in water, giving a pale yellow solution, but is without measurable optical activity. The Molisch reaction is intense, the biuret reaction negative and the orcinol test gives merely a greenish-red colour quite different from the reaction of glycuronic acid or the pentoses. Solutions of the substance do not reduce Fehling's solution but do so after hydrolysis with acid.

On account of these properties it was thought desirable to obtain an accurate determination of the molecular weight. The cryoscopic method was used, the solvent being water, and the following results obtained:

Carbohydrate from globulin.

$c = 6{\cdot}406$; $\Delta = 0{\cdot}348°$, $0{\cdot}347°$, $0{\cdot}350°$; Mean $0{\cdot}348$. $M = 340{\cdot}6$.

A disaccharide $C_{12}H_{23}O_{10}N$ would have $M = 341$. The substance is correctly represented, therefore, by its empirical formula.

Identification of the component sugars. An attempt was made to isolate the constituent sugars in the following way. 0·595 g. substance (from serum-globulin) was heated with 18·3 cc. of 20 % hydrochloric acid for 6 hours in

[1] All microanalyses were performed either by Dr Ing. A. Schoeller of Berlin or by Mr A. R. Colwell of the Biochemical Laboratory, Cambridge.

a sealed tube at 100°. The solution contained some dark tarry matter which was filtered off and the filtrate concentrated *in vacuo* over sulphuric acid and sodium hydroxide until nearly dry. Some crystalline material which separated was removed, washed with a little alcohol and dried for analysis. It proved to be a hydrochloride containing nitrogen.

Analysis (micro):	C	H	N	Cl
Found	33·02	6·41	6·25	16·31
Calcd. for $C_6H_{14}O_5NCl$ ... ... (glucosamine hydrochloride)	33·40	6·54	6·50	16·45

A solution of this substance when heated on the water-bath for 2½ hours with phenylhydrazine hydrochloride and sodium acetate yielded an osazone which was identified as glucosazone microscopically and by its melting point 202°–206°. It was concluded therefore that the substance was *glucosamine hydrochloride*. The yield was 0·122 g. or 41 % of the theoretical.

The residual solution from which the glucosamine had separated was evaporated to dryness and the residue repeatedly extracted with alcohol, the alcoholic extracts being combined, and the alcohol evaporated on the water-bath. The residue was taken up in a little water and to this solution phenylhydrazine hydrochloride and sodium acetate were added and the mixture left in a boiling water-bath. An osazone formed resembling glucosazone in microscopical appearance. The crystals were washed with 20 % alcohol then dissolved in hot absolute alcohol; water was added until turbidity resulted and the solution allowed to cool. Typical glucosazone crystals formed, having melting point 204°–205°. It was thus shown that the sugar associated with glucosamine in this carbohydrate derivative obtained from serum-globulin was a hexose capable of yielding glucosazone and therefore either glucose, fructose or mannose.

Reducing power and optical activity of hydrolysate.

In order to gain further evidence concerning the nature of the hexose some periments were experformed in which the carbohydrate material was hydrolysed by boiling with acid and the optical rotation and reducing power of the solution determined when hydrolysis was complete.

The method employed for determining reducing power was that of Willstätter and Schudel [1918] where the active agent is sodium hypoiodite which distinguishes between aldoses and ketoses, only the former being oxidised. 0·4689 g. of the carbohydrate from globulin was dissolved in 50 cc. of *N* HCl and the mixture boiled. At intervals, samples were taken and reducing power determined by the iodine method.

Time (hours)	Reducing power expressed as glucose (mg.)
0	—
½	78·7
1	229·0
2	425·4
3½	459·7
5	481·2
Theoretical possible	493·5

Within 5 hours hydrolysis was practically complete, the reducing power being 97·5 % of theory (all expressed as glucose). The remaining solution, amounting to 14 cc. in all, was neutralised by sodium hydroxide, filtered and its rotation observed.

Rotation in 2 dm. tube	$= + 0{\cdot}40°$
Concentration (from reducing power)	$= 567{\cdot}3$ mg./100 cc.
$[\alpha]^{18°}_{5461}$	$= + 35{\cdot}28°$.

Assuming glucosamine and mannose to be present in equal proportions, calculation from the known constants gives, for the mixture, an estimated[1] $[\alpha]_{5461}$ of $+ 38{\cdot}64°$, whilst for glucosamine + glucose $[\alpha]_{5461} = + 61{\cdot}27°$. Considering the difficulties of measurement, using such small quantities, the agreement is sufficiently close. It may be pointed out that since both components of the disaccharide are oxidised in the iodine method the possibility of fructose being present is excluded. The substance must therefore consist of glucosamine together with either glucose or mannose and the above polarimetric evidence strongly suggests that the hexose is mannose.

Isolation of mannosephenylhydrazone.

For the isolation of mannosephenylhydrazone from the hydrolysis mixture 1 g. carbohydrate material was boiled with 10 cc. *N* HCl for 5 hours. When cold, the brownish-yellow liquid was neutralised to litmus paper by the very cautious addition of sodium hydroxide solution. It was then filtered and warmed to about 37°, and solid phenylhydrazine hydrochloride (0·4 g.) and crystalline sodium acetate (0·38 g.) were added. Solution took place very rapidly, whereupon the liquid was again filtered, allowed to cool and gently stirred as soon as crystals began to separate. The tube was stoppered and left in ice overnight. The crystalline material (which consisted of extremely fine microscopic prisms or needles) was separated by centrifugation and washed repeatedly, in the same manner, first with distilled water, then with cold 60 % alcohol and finally desiccated over sulphuric acid *in vacuo*.

By elementary analysis and melting point it was identified as mannosephenylhydrazone. Preparations contained a little ash (about 0·9 %), which has been allowed for in calculating the percentages.

Analysis (micro):		C	H	N
Carbohydrate from globulin. Found	...	52·95	6·78	10·85
Carbohydrate from albumin. Found	...	53·11	6·54	10·82
Calcd. for mannosephenylhydrazone ($C_{12}H_{18}O_5N_2$)	...	53·33	6·67	10·37

The substances had M.P. 198–199° and 199–200° (uncorr.) respectively. The values given in the literature for the melting point of mannosephenylhydrazone vary from 195° to 200°. A specimen prepared from pure mannose had M.P. 200°; mixed M.P. with substance isolated 199°.

[1] The factor 1·18 has been used to convert values of $[\alpha]_D$ into those for the line 5461 Å.

Isolation of glucosamine hydrochloride from the carbohydrate obtained from albumin.

The carbohydrate materials isolated from the albumin and globulin respectively agree in having the same elementary composition and molecular weight and also in their physical and general properties. They appear in fact to be identical. Glucosamine was identified in the substance from albumin in the following manner.

0·2 g. of the carbohydrate was boiled for 5 hours with 50 cc. of 10 % hydrochloric acid. The resulting solution was concentrated over sulphuric acid and sodium hydroxide *in vacuo* until crystals began to separate. After standing overnight the crystals were separated from the dark syrupy mother liquor, washed with 97 % alcohol and then dried. They were yellowish white in colour, but after recrystallisation from dilute hydrochloric acid were obtained colourless.

Analysis (micro):	C	H	N	Cl
Found	33·43	6·92	6·49	16·96
Calcd. for glucosamine hydrochloride ($C_6H_{11}O_5NCl$)	33·40	6·54	6·50	16·45

Preparation of the carbohydrate material by tryptic hydrolysis of the protein.

The carbohydrate groups present in the mucins and other glucoproteins have the structure of polysaccharides in which glucosamine and glycuronic acid are united. It has been shown by Levene and La Forge [1913], however, that these substances also contain sulphur, in the form of sulphuric acid in ester linkage with the sugars, whilst the amino-group of the glucosamine is not free, but is acetylated. Since the conditions of hydrolysis obtaining in the work here recorded were such as would probably remove any ester-linked sulphuric acid, were this originally present in the carbohydrate complex obtained from the serum-proteins, and also such as to eliminate any acetyl groups from their union with amino-nitrogen, it was felt that possible criticism should be met by endeavouring to obtain the carbohydrate by other methods.

As identical substances were obtained from the albumin and globulin respectively of horse-serum, in this experiment their separation was omitted. In all other details the preparation of the proteins resembled that previously described, but addition of ammonium sulphate was carried to full saturation and the albumin and globulin filtered off together.

200 g. of the carefully purified and dried mixed serum-proteins were suspended in 2 litres of water together with a little toluene. After standing overnight the protein was soft and evenly dispersed throughout the liquid. Sufficient sodium hydroxide to bring to p_H 8·3 was then added, followed by 10 cc. of Allen and Hanbury's "Trypsin." This enzyme preparation was selected as being the most suitable since it gave an almost entirely negative Molisch reaction; other commercial preparations contained considerable quantities of carbohydrate material. After 2 days' incubation at 38° a further 10 cc. of enzyme solution was added and the p_H readjusted to 8·3.

Hydrolysis was allowed to proceed at 38° for 4 weeks, the mixture being shaken occasionally. It was then filtered through pleated filter papers. The filtrate was clear yellow in colour and gave an intense Molisch reaction. A scheme of isolation was followed identical with that previously described. Much trouble was encountered in the final stages, however, in ridding the carbohydrate of admixed peptone material, and a repetition of the precipitation with lead acetate and ammonia, followed by all the subsequent stages, was necessary before satisfactory analytical figures were obtained. These showed the substance to be identical with that isolated by the baryta hydrolysis method. The yield was slightly smaller (about 1·5 %).

Sulphur was tested for but was found to be absent from the preparation.

Analysis (micro):	C	H	N
Found	42·68	7·39	4·65
Calcd. for $C_{12}H_{23}O_{10}N$	42·21	6·79	4·10

It is clear as a result of these experiments that the carbohydrate substance present in the serum-proteins is of a type quite different from that occurring in the mucins and has nothing in common with the latter save the presence in each of glucosamine as one of the constituent units.

Structure of the carbohydrate derivative.

The molecular weight of the substance isolated is in accordance with that required by the simple formula $C_{12}H_{23}O_{10}N$. Hence it is clear that the substance is of the disaccharide type and not a complex polymer. The absence of reducing properties would in this circumstance suggest a linkage similar to that present in trehalose, involving the two reducing groups of the component sugars.

On the other hand, á union of the α- or β-glucoside type would result in a molecule exhibiting the same behaviour towards copper solutions if, at the same time, the reducing group of one or other of its components was involved in some way—for example, with the amino-group of the glucosamine—so that its potentialities were in abeyance.

In order to gain further insight into the constitution of the compound attention was directed towards the behaviour of the amino-group. Glucosamine and its methyl ester give off nitrogen quantitatively when acted upon by nitrous acid. When the disaccharide was investigated, using the Van Slyke microapparatus, the following results were obtained, from which it is clear that the amino-group, though slowly attacked, is capable of reacting quantitatively to form nitrogen.

Solution (*a*). 11·768 mg. in 2·5 cc.

Reaction time 3 mins. ... 1·77 cc. gave 0·22 cc. N_2 at 763 mm. and 20° = 1·66 % N_2.

Solution (*b*). 50 mg. in 10 cc.

Reaction time ½ hr. ...	2 cc. gave	0·41 cc. N_2	at 769 mm.	and	19°	= 2·37 % N_2
„ 1 „ ...	2	0·56	765	„	20	= 3·23 „
„ 2 „ ...	2	0·65	762	„	21	= 3·67 „
„ 6 „ ...	2	0·70	760	„	20	= 3·99 „
				Theoretical		= 4·10 „

Hynd and Macfarlane [1926] have recently examined the behaviour towards nitrous acid of a number of amino-compounds. Glucosamine they found to react only slowly in presence of 20 % acetic acid, taking 18 hours for complete decomposition, although its salts like the hydrochloride, or the free base in presence of mineral acid, yielded all their nitrogen as gas within half an hour at 20°.

They conclude from these observations that glucosamine must contain the modified betaine ring suggested by Irvine and Hynd [1913], whilst in the case of its salts, or of the base itself in presence of mineral acid, this linkage is unstable and gives way to form the free amino-group.

In many ways the properties of the carbohydrate compound are in keeping with the suggestion that the nitrogen atom of the glucosamine is involved in a ring of the betaine type, the other hexose being attached in a manner similar to the methyl group in Irvine and Hynd's α-aminomethylglucoside. Like this substance it exhibits a stability towards acid hydrolysis that is quite irreconcilable with a simple glucosidic linkage, and the slowness with which the reaction takes place between the amino-group and nitrous acid in the presence of a weak organic acid like acetic acid suggests that the substance belongs to the "free-glucosamine" type, containing ring-nitrogen rather than that of the glucosamine salts as typified by the hydrochloride.

It is impossible, however, to overlook a point of some difficulty arising at this juncture, should the suggestion be accepted that the nitrogen atom is part of a betaine ring.

The work of Kenner and Earl [1927] has shown that a grouping, in which a nitrogen atom occurs in the quinquevalent state, bears a positive charge. In most cases this will give rise to a negatively charged ion but in the case of a disaccharide such as that described here it is difficult to see how this condition could be fulfilled. It is, however, noteworthy that in all preparations a very great difficulty was encountered in attempting to eliminate traces of chlorine from the compound during its purification, an attempt which, in fact, proved unsuccessful. There was in each case a small quantity of chlorine, amounting to some 2 to 4 %, which could not be removed by the precipitation procedure adopted. This quantity is smaller than would be required by one atom of combined chlorine and was moreover variable, but its presence accords with the view that there was resident somewhere in the molecule a sphere of electropositive influence.

Experiments were next undertaken to determine whether any of the usual enzymes acting on carbohydrates were capable of hydrolysing the compound. Reducing power was determined by the method of Hagedorn and Jensen, at intervals of time up to 4 days. The enzyme preparations were all freshly prepared and the activity of each was tested upon a suitable substrate. In all cases buffer solutions of the appropriate p_H were added to the test and control solutions.

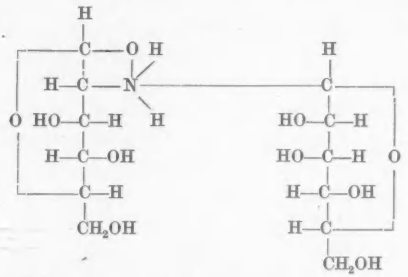
The following results were obtained:

Enzyme	Effect
Emulsin	Nil
Ptylain	Nil
Pancreatic amylase	Nil
Maltase	Slight hydrolysis?

With maltase the observed increase in reducing power was but slightly greater than the experimental error; trehalase, unfortunately, has not yet been available for trial.

The evidence furnished by these enzyme experiments militates against the view that an α- or β-glucosidic linkage is present. It is in keeping with the suggestion that the two sugar molecules are united as in trehalose, or as in the modification of this linkage possible when one constituent is an amino-sugar, in which the betaine ring occurs.

In the absence of further evidence it is perhaps unwise to consider the structure of this carbohydrate as proven. The following formula is, however, put forward with a certain degree of confidence as indicating its probable constitution.



There are two possible ways in which such a structure could enter into combination with the amino-acid complexes of the protein molecule. In the first place attachment might occur through the nitrogen atom, or, secondly, it is possible that condensation might occur between one or other of the hydroxyl groups and some constituent of the protein molecule. In the absence of further knowledge there is no way of deciding between these two alternatives.

Deductions concerning the molecular weights of the serum-proteins.

Both the albumin and globulin of horse-serum yielded about the same quantity of pure carbohydrate material, namely 2 %. Since the carbohydrate possesses a molecular weight of 341, the minimal molecular weight of each protein is given by the expression $M = \frac{341 \times 100}{2} = 17050$. Of course such a calculation can give only approximate figures since it is not possible to determine the percentage content of carbohydrate more exactly than by direct isolation; however, this figure is in quite good agreement with the values recorded in the literature and found by methods very different from that here employed [see Svedberg and Sjögren, 1928; Cohn, Hendry and Prentiss, 1925].

Discussion.

The demonstration that a carbohydrate complex is one of the structural units present in both the albumin and globulin of horse-serum is important and raises several problems of biological, as well as of chemical, interest. It is somewhat surprising to find that this new constituent is present in relatively large amounts—approximately 2 % of the dry weight of each protein—and one naturally wonders whether any particular biological function is subserved by its presence. Reference will be made later to the work of Glassmann [1925, 1926].

The part which the plasma-proteins play in immunity reactions has been the subject of many researches. In view of the very interesting results which have been obtained recently in a related branch of immunology by Heidelberger and Avery and other workers it is desired to call attention to the fact that the soluble specific substances obtained by these authors from pathogenic bacteria have been shown to be carbohydrate derivatives of a relatively simple structure. Many of these specific substances contain nitrogen, presumably as the amino-group of an amino-sugar.

It is so suggestive to find carbohydrate structures having a complexity at least comparable with that of the carbohydrate present in the serum-proteins and possessed of specific immunological properties, that, whilst avoiding undue emphasis, the present writer would suggest that future investigations concerning the immunological rôle of the plasma-proteins should not overlook the fact that both albumin and globulin contain carbohydrate material as an integral constituent of their molecules.

Returning to purely chemical issues, the question arises of the relationship existing between the serum-proteins and the class of conjugated proteins known as glucoproteins. The work of Levene and La Forge [1913] has shown that the mucins contain a carbohydrate complex which is quite different from the disaccharide complex of the serum-proteins. Moreover, the relations existing between the carbohydrate and the serum-proteins are not those usual between prosthetic group and protein moiety; the carbohydrate appears to enter into their structure just as an amino-acid would or as the phosphorus-containing complex enters into the structure of caseinogen [see Rimington, 1927].

Throughout the course of the present research attention has been confined strictly to the two proteins derived from horse-serum. It is hoped, at a later date, to extend and develop the work and, as a preliminary result, it has been ascertained that a carbohydrate substance is present also in wool-keratin. Whether or not it will prove to be the case that carbohydrate complexes are of general occurrence in the protein molecule it is impossible to say, but recently Fränkel and Jellinek [1927] claimed that a carbohydrate composed of glucosamine and mannose occurred in egg-albumin. Unfortunately these authors did not determine the molecular weight of this product, neither did they investigate its structure. Whilst there are points of resemblance between

the disaccharide isolated by me from serum-proteins and the substance obtained by Fränkel and Jellinek, in other characteristics it is notably different. Thus Fränkel and Jellinek state that their preparation was hydrolysed by ptyalin, and furthermore that it yielded no nitrogen gas when treated with nitrous acid in the Van Slyke apparatus. The substance obtained by me from serum-proteins was not attacked by any of the carbohydrase preparations—including ptyalin—which were allowed to act upon it. Its nitrogen, on the other hand, is evolved completely though slowly as nitrogen gas in the Van Slyke apparatus.

Soon after this work was completed Dische [1929] reported some investigations upon the "protein-bound" sugar of the blood. His interest appears to centre round the clinical significance of protein-bound sugar, but by adding phenylhydrazine hydrochloride and sodium acetate to an acid hydrolysate of whole plasma which had been treated with phosphotungstic acid, he succeeded in isolating mannosephenylhydrazone and also in detecting the presence of another, non-fermentable, reducing substance which, he considers, may be glucosamine.

The conditions employed by Dische were such as would hydrolyse not only the plasma-proteins but also the glucosamine-mannose disaccharide which would be obtained, during the course of the hydrolysis, from the albumin and globulin present.

Since, largely owing to the work of Glassmann, the whole subject of protein-bound sugar has recently taken on a new clinical significance, it is of interest to notice how the present research illuminates several long disputed points. Lèpine [1918], to whom the conception of "bound sugar" is due, considered that this was sugar held in glucosidic linkage. Bierry and Fandard [1911], on the other hand, rejected this view since they found that neither emulsin nor invertase increased the reducing power of plasma, boiling acid alone being capable of so doing. The present findings clearly substantiate Bierry and Fandard's views. Secondly, the values obtained by different authors for the proportion of bound sugar have not always agreed. Thus Glassmann [1925, 1926] arrived at figures six or seven times as large as those of Bierry. Dische has shown that alkaline mercuric nitrate—employed as a protein precipitant by Bierry—removes some reducing material from solution. In the light of the present investigations it is clear that the material so lost was glucosamine derived from the glucosamino-mannose.

There remains for discussion one point more; namely, the chemistry of humin formation. Gortner [1916 and elsewhere] has shown that the humin produced from proteins undergoing hydrolysis by boiling acids is derived from an interaction between tryptophan and some substance, most probably of an aldehydic nature, formed together with the amino-acid during the hydrolysis of the protein. Glucose, starch and other carbohydrates were shown to cause an increase in the yield of humin when added along with the protein for hydrolysis. Nothing further is known concerning humin formation,

but the discovery of a carbohydrate complex in two proteins at least, and in relatively large amount, opens up a new field for experimental attack. This carbohydrate may be the precursor of the aldehyde reacting with tryptophan, and the failure of investigators in the past to appreciate its presence is no doubt due in large part to the fact that acid hydrolytic agents were customarily employed in any research upon the nature of the constituent units of the protein. The bulk of the carbohydrate would thereby be lost as a result of secondary reactions.

A systematic examination of these and allied problems is contemplated in the near future. So also is a thorough examination of the different classes of proteins with a view to ascertaining whether the nitrogenous disaccharide described in the above pages is peculiar to the proteins of horse-serum or whether it, or some related compound, occurs universally as a structural unit of protein material.

Summary.

1. From the carefully purified proteins of horse-serum, a carbohydrate derivative has been obtained which is regarded as a definite structural unit of these proteins.

2. The substance isolated has the composition $C_{12}H_{23}O_{10}N$, is devoid of reducing properties and exhibits in solution no measurable optical activity.

3. The constituent sugars have been identified as glucosamine and mannose and it has been shown that the compound has a molecular weight corresponding to that required by the simple disaccharide formula. Identical products are obtained from serum-albumin and globulin respectively and in a yield of approximately 2 % in each case.

4. Calculation from the yield gives figures for the minimal molecular weights of the proteins which are in good agreement with values previously recorded in the literature.

5. Exactly the same product has been obtained from the serum-proteins when trypsin was employed as the hydrolytic agent, thereby establishing the distinction between this carbohydrate and the sulphur-containing tetrasaccharide of the glucoproteins.

6. A structure has been suggested for the carbohydrate substance.

7. Some chemical considerations, arising out of the occurrence of carbohydrate material in these two proteins, have been discussed in connection with physiological and immunological problems.

My thanks are due to the Research Control Committee of this Association for their permission to conclude and publish this research, the greater part of which was carried out at Cambridge. I also wish to thank Sir F. G. Hopkins for his continued interest in this problem and Mr A. Colwell of the Cambridge Biochemical Laboratory for his kindness in carrying out for me a very large number of micro-analytical estimations.

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LII. ON THE NATURE OF THE CARBOHYDRATES FOUND IN THE JERUSALEM ARTICHOKE.

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In spite of repeated investigations, divergencies still exist as to the nature and relative proportions of the carbohydrates found in the tubers of the Jerusalem artichoke (*Helianthus tuberosus*).

The most detailed account of these carbohydrates has been given by Tanret [1893], who carried out investigations with tubers gathered in September and October. Tanret pressed these tubers in a press, collected the juice, and mixed it with about one-tenth its volume of warm basic lead acetate solution of specific gravity 1·25. After filtering off the precipitate formed, the excess lead in the filtrate was removed by means of the requisite amount of dilute sulphuric acid. Addition of hot concentrated baryta water to the clear filtrate thereby obtained yielded a precipitate, which Tanret describes as the barium compound of inulin. Further precipitates could be obtained from this baryta-containing filtrate by addition of increasing quantities of alcohol. Altogether seven carbohydrates were separated from the artichoke juice (see Table I).

Table I.

	Percentage of carbohydrates[1]	
	Calculated on dry tubers	Calculated on total carbohydrates
Inulin	3·12	20·62
Pseudo-inulin	0·35	2·31
Inulenin	1·25	8·26
Helianthenin	0·83	5·49
Synanthrin	7·26	47·98
Sucrose	1·78	11·77
Laevulose and dextrose	0·54	3·57

[1] In his paper Tanret gives his results in grams of carbohydrates per litre of concentrated juice. His figures have been converted as above for the purpose of comparison with the results of other workers.

The reducing sugars laevulose and dextrose were present in small quantities only. The relative proportions of the various other carbohydrates are not given by Tanret, except in the case of a sample of juice pressed from tubers which had been collected in the autumn, but stored in a cellar till the following June before analysis. In consequence of the prolonged storage of these tubers the figures given by Tanret may not be correct; they are quoted, however, as they represent the most complete analysis available in the existing literature.

Other investigators have arrived at different conclusions with regard to the amount of inulin present in artichoke tubers. Thus Konig [quoted by Czapeck, 1913], reports the presence of 58 % of inulin, presumably on the total carbohydrates present; while Shohl [1923] records 15·5 % of inulin in the fresh tubers.

The presence of sucrose in the tubers has been reported by Dubrunfaut [1867] and by Colin [1918]. Helianthenin and synanthrin are names given by Tanret to two laevorotatory carbohydrates with the following characteristics.

Table II.

	Specific rotation $[\alpha]_D^{20°}$	Melting point	Solubility in water	Specific rotation after inversion $[\alpha]_D^{20°}$
Helianthenin	−23·5°	176°	50 %	−70·2°
Synanthrin	−17°	170°	in all proportions	−70·6°

Pseudo-inulin and inulenin are closely related to the inulin proper, but are stated to differ from it in their specific rotations, which are said to be for pseudo-inulin − 32·2°, and for inulenin − 29·6°. On inversion the carbohydrates show a specific rotation of − 85·6° and − 84° respectively.

Of all the carbohydrates isolated by Tanret from the artichoke tubers only sucrose and dextrose give a positive specific rotation. It is to be expected, therefore, that the juice obtained by extracting the fresh tubers with water will show a negative rotation. That the juice is laevorotatory is confirmed by Colin [1918], who finds that an extract of the tubers is strongly laevorotatory when prepared in autumn (September). When taken from tubers collected during early spring, Colin obtained dextrorotatory juice. This undoubtedly implies that considerable changes have occurred in the carbohydrates, changes which, according to Colin, result in an altered fermentability of the tubers. Colin points out that it is necessary to subject autumn-gathered tubers to a preliminary hydrolysis with acids before they can be completely fermented by yeast, while the carbohydrates of the tubers collected in spring may be directly converted into alcohol by yeast without preliminary inversion. That a preliminary hydrolysis of the tubers raises the yield of alcohol obtainable from them has also been observed by Rudiger [1920] and by Windisch [1920].

On the assumption that inulin, which is unfermentable by yeast in the pure state[1], is really present in artichoke tubers, the above observations would

[1] An observation which has been experimentally confirmed by the writers.

seem to indicate that inulin undergoes an inversion during storage of the tubers, resulting in its conversion into a fermentable sugar; unless it be considered justifiable to assume with Tanret [1893] that inulin is fermentable by yeast when present in an already fermenting mixture of other carbohydrates. The experiments on which Tanret bases this assumption were carried out with tubers gathered during the early autumn, but stored in a cellar until June of the following year. In the juice obtained from these tubers Tanret found as much as 1·36 % of inulin, calculated on the tubers, of which he was able to recover only 27·5 % after fermentation of the juice by a yeast, 72·5 % of the original amount having disappeared during fermentation.

As certain investigations carried out by the writers made it desirable to collect further information on the fermentative properties of the carbohydrates of the Jerusalem artichoke, it was thought justifiable to repeat part of the work referred to above and to endeavour to co-ordinate the divergent views of previous workers.

As a preliminary it was decided to determine the rotation of juice pressed from tubers collected both during autumn and spring. The results obtained are given in Table III and show that a change from laevo-rotation to dextro-rotation does take place. The actual figures when expressed as specific rotations of the whole mixture of carbohydrates found in the tubers agree closely with the observation of Colin [1918], who observed a specific rotation not exceeding + 10° in tubers stored until Feb.–March. The comparatively strong positive rotation of the carbohydrates in spring juice reaches its maximum about March and has decreased to + 2·12° by May, when germination has proceeded for some time and emptied the tubers of a considerable percentage of their carbohydrates.

Table III.

Date of examination	Percentage of carbohydrates		Observed rotation of juice $[\alpha]_D^{20°}$		Specific rotation of carbohydrate mixture in juice $[\alpha]_D^{20°}$	
	Total	Reducing	Before inversion	After inversion	Before inversion	After inversion
3. xi. 23	3·2	—	−0·095°	−3·13°	−1·48°	−58·9°
16. xii. 23	5·16	0·64	−0·13°	−6·23°	−1·26°	−61·6°
17. i. 24	(*a*) 3·43 (*b*) 4·29	(*a*) *0·31	(*a*) +0·17°	(*b*) −4·21°	+1·98°	−61·4°
27. ii. 24	6·19	0·33	+0·71°	−3·58°	+6·06°	−57·84°
18. iii. 24	5·65	0·41	+0·59°	−0·79°	+4·49°	−57·1°
7. v. 24	5·32	—	+0·261°	−0·764°	+2·12°	−58·6°

* Two different juices (*a* and *b*) were used for this determination.

It should be noted that the percentage of reducing sugars in the juice before inversion is very small, even in samples of juice extracted from tubers stored until March. No increase or only a very slight increase in the laevulose and dextrose content has therefore occurred during storage. This is interesting, as it shows that the change in the rotation is not due to an increase in the concentration of a dextrorotatory reducing sugar.

In order to investigate the reason for this change it was decided to attempt the isolation of the various carbohydrates present both in autumn and spring tubers. To do this the method recommended by Tanret was adopted. His method, however, was not found easy to carry out[1], and after several unsuccessful attempts it was decided to proceed with the separation in a somewhat different way, though still adhering to Tanret's method of utilising alcohol of varying strengths for the separation of the individual fractions. The procedure finally adopted was as follows. The freshly-dug tubers were carefully washed and then minced to a fine pulp. The pulp was mixed with its own weight of warm distilled water and the mixture kept at 60°–70° for half an hour. It was then pressed and the juice collected. Basic lead acetate solution of SP.G. 1·25, sufficient to clarify the juice, was added to the extract and the excess lead removed from the clear filtrate with sulphuric acid and sulphuretted hydrogen. The clear filtrate was concentrated *in vacuo* at a temperature not exceeding 42°. Its carbohydrate content was thereby increased to about 20 %. To every 100 cc. of this solution one litre of 95 % ethyl alcohol was added, and the mixture well shaken. The alcohol caused a voluminous precipitate to form when juice obtained from autumn tubers was used. This precipitate was collected on a filter, washed with 95 % alcohol, with ether, and finally dried *in vacuo* over sulphuric acid. When dry it formed a snow-white, light and almost tasteless powder.

For the further purification of this powder it was redissolved in as little warm water as possible, and 95 % ethyl alcohol added to the cooled solution until its alcohol concentration amounted to 50–55 %. The precipitate, after standing overnight, was collected and dried as described above. To the filtrate a further quantity of 95 % alcohol was added to make its concentration equal to about 85 % ethyl alcohol. A precipitate was again formed, which, when dried as already described, formed a granular, sandy-coloured powder. After precipitation of the above two fractions (B and C) the filtrate contained only traces of carbohydrates.

The filtrate from the mixture of clarified juice with alcohol was evaporated *in vacuo* (temperature 42°) to a thick syrup, containing between 40–45 % of carbohydrates. The isolation of these carbohydrates was at first found almost impossible. Finally, however, the following method separated the syrup into two distinct fractions. Three g. of the syrup were poured into 80 g. of absolute alcohol, containing not less than 99·5 % of ethyl alcohol, and the mixture was stirred vigorously for some time. Part of the syrup was thereby converted into a flocculent precipitate, which could be filtered off, while another part formed a gummy substance on the bottom of the container, a third part remaining dissolved in the alcohol. The gum, after dissolution in a very small quantity of water, could again be divided into a flocculent precipitate and

[1] Similar difficulties are also reported by Pringsheim and Lassmann [1922], who were unable to confirm Tanret's results as regards the presence of pseudo-inulin, inulenin, synanthrin and helianthein.

a gum by the addition of the required quantities of absolute alcohol. Repeating this process a sufficient number of times the whole of the syrup could be converted into a flocculent precipitate (fraction D), and a carbohydrate (fraction E) remaining dissolved in this strong alcohol. This latter fraction could be precipitated by the addition of ether to its alcoholic solution, and on separation, formed a gummy and extremely hygroscopic substance, light brown in colour and having a very sweet taste. The flocculent precipitate was not hygroscopic, though extremely soluble in water. It was also light brown in colour and had a sweet taste. The filtrate from the fourth fraction (E) contained traces only of carbohydrates.

The whole of the carbohydrates originally present in the juice had therefore been isolated by the above process.

Subjecting both autumn and spring tubers to the treatment described above the following yields of carbohydrates were obtained (Table IV).

Table IV.

		Juice prepared from tubers dug and extracted Nov. 1923		Juice prepared from tubers dug and extracted March 1924	
Fraction	$[\alpha]_D^{20°}$	Obtained by addition of alcohol	Present as percentage of total carbohydrates	Obtained by addition of alcohol	Present as percentage of total carbohydrates
B	−34·96°	Insoluble in 55 % alcohol	28·04	Insoluble in 55 % alcohol	1·02
C	−33·42°	Insoluble in 85 % alcohol	13·40	Insoluble in 85 % alcohol	
D	Inactive to −1·233°	Insoluble in 98 % alcohol	38·00	Insoluble in 98 % alcohol	44·7
E	−2·91° in autumn to −24·91° in spring	Insoluble in a mixture of ether and 98 % alcohol	20·56	Insoluble in a mixture of ether and 98 % alcohol	54·28

This table is of considerable interest in several respects. It shows, for instance, that there is a very material difference in the distribution of the carbohydrates in autumn and spring tubers, the latter containing less laevorotatory and less insoluble substances than the former. Further, the table appears to explain why artichoke tubers have in some cases been found to contain inulin, while in other cases inulin has been absent.

As seen from Table V fraction B corresponds with the inulin of Tanret; fraction C may be identical with Tanret's pseudo-inulin. These fractions, B and C, disappear from the tubers toward spring and give place to the more readily soluble fractions D and E.

Fractions D and E do not compare with any of Tanret's fractions. They are probably mixtures of carbohydrates, since their specific rotations vary, depending on the season at which they are isolated from the tubers. As both of these fractions yield large percentages of glucose on hydrolysis, the possibility that one of their components is sucrose cannot *a priori* be excluded,

particularly in view of the fact that both Dubrunfaut, Colin and Tanret have isolated sucrose from Jerusalem artichokes.

Table V.

Properties		Inulin	Fraction B	Pseudo-inulin (Tanret)	Fraction C
Specific rotation	Before inversion	−38° to −39°	−34·26°	−32·2°	−33·12°
	After	−85·7°	−83·42°	−85·6°	−77·94°
Reduction of Fehling's solution		No reduction	No reduction	No reduction	No reduction
Solubility in water		Slightly soluble	Slightly soluble, about 0·062 % at 12°	More soluble than inulin	Readily soluble
Melting point		165°–178°	No sharp melting point between 158–163°	Decomposes at 175°	
Effect on ammoniacal silver nitrate		Reduced	Reduced	—	—
Behaviour towards iodine		Yellow coloration	Yellow coloration	—	—
Melting point of osazone after inversion		205°	205°	—	—
Appearance		White flocculent powder, tasteless	White flocculent powder, tasteless	—	Almost white to light fawn-coloured powder with slightly sweet taste

Attempts have repeatedly been made by the writers to isolate sucrose from these fractions D and E, but complete success has not yet been attained. There are good reasons for supposing, however, that fraction E is a mixture of a small proportion of D with a large proportion of sucrose, while fraction D, as isolated by the above treatment, contains a small quantity of sucrose. If it may be assumed that the whole of the dextrose found in the inverted samples of fractions D and E has been derived from sucrose, the following specific rotations would have to be attributed to the remaining components (Table VI, column 4).

Table VI.

Fraction	Specific rotation of fraction calculated from observed rotation	Proportion of dextrose and laevulose in the inverted fraction	Assuming the dextrose found to have been derived from sucrose the remaining compounds of the fractions would have sp. rotation
D from autumn tubers	Zero	22·8 % dextrose 77·4 % laevulose	−50·94°
D from spring tubers	− 1·23°	23·6 % dextrose 76·4 % laevulose	−56·37°
E from autumn tubers	− 2·91°	27·9 % dextrose 72·1 % laevulose	−69·0°
E from spring tubers	−24·91°	31·7 % dextrose 68·3 % laevulose	−38·3°

In every case this specific rotation is more negative than that of inulin, probably signifying that the compounds in question are hydration products of inulin, possibly condensation products of laevulose with 6 or 3 laevulose groups, instead of 9 as in the case of inulin [Pringsheim and Aronowsky, 1922].

The question of the fermentability by yeast of the various carbohydrate fractions isolated from the tubers of the Jerusalem artichoke was investigated in a series of experiments the results of which are given in Table VII.

The carbohydrates were dissolved to a concentration of 4 % in a water extract of maize meal, made by digesting 7 parts of maize meal with 100 cc. of physiological saline at 37° for 16 hours in presence of 1 cc. of toluene per litre. The liquid was filtered through an ordinary filter-paper and was then boiled to remove any traces of toluene present[1]. The solutions were sterilised by fractional sterilisation in order to avoid the decomposition of the sugars. After inoculation the samples were incubated at 35° for 4 and 10 days respectively before distillation.

The yeast used in the experiments was a pure culture distillery yeast fermenting well at 35°.

An almost complete conversion into ethyl alcohol was obtained with fraction D and E of both autumn and spring tubers. Fractions B and C, on the other hand, remained unfermented.

Table VII.

Fraction	Carbohydrate concentration of solution to be fermented %	Carbohydrate concentration in mash after fermentation %	Duration of fermentation days	Yield of alcohol obtained calculated on carbohydrates present %	Yield obtained: percentage of that theoretically possible
B	4·2	4·04	10	Traces	—
C	4·2	3·85	10	Traces	—
D autumn tubers	4·2	0·47	4	42·5	88·8
D spring „	4·2	0·39	4	43·6	91
E autumn tubers	4·2	0·23	4	44·5	94·6
E spring „	4·2	0·23	4	44·5	94·6
Controls—					
Sucrose	4·2	Less than 0·04	4	47·6	99·1
Laevulose	4·2	Less than 0·03	4	49·5	99·3
Dextrose	4·2	Less than 0·03	4	49·5	99·3

The non-fermentability of the B and C fractions agrees well with what would be expected from a carbohydrate of the nature of inulin, though it makes it rather difficult to explain certain earlier experiments and the fermentation experiments of Tanret.

Tanret in his investigations found that 75 % of the inulin present in the tubers used disappeared during the fermentation, and he suggested that inulin might at least be partly fermentable in the presence of readily fermentable

[1] The extract (after inversion) contained less than 0·2 % carbohydrates in solution.

compounds. A number of experiments have been carried out to determine how far this may be the case, and mixtures of two or more of the fractions isolated from autumn-gathered artichoke tubers have been subjected to fermentation by yeast in the manner already described. Table VIII illustrates the results obtained.

Table VIII.

Fraction	Carbohydrate concentration of mash before fermentation %	Carbohydrate concentration of mash after fermentation %	Duration of fermentation days	Yield of alcohol obtained calculated on carbohydrates present %	Yield of alcohol obtained calculated on the fractions respectively: B+C %	D %	E %
B+C	4·2	3·99	10	2·7	2·7	—	—
B+C+D	4·2	2·42	10	20·4	2·7	38·12	—
B+C+D+E	4·2	1·51	10	33·0	15·3	43·6	44·5
D	4·2	0·47	4	43·6	—	43·6	—
E	4·2	0·23	4	44·5	—	—	44·5

The fractions were present in the proportions in which they were found in the tubers.

It will be seen that a higher yield of alcohol was obtained in the mash where all four fractions were mixed than should have been obtained had the B + C fraction not been attacked. It is true that the yields of alcohol from the fractions D and E are estimated at 43·6 % and 44·5 % respectively. Yet even assuming that both of these gave yields of 50 %, or the absolute maximum obtainable, the alcohol collected from the mixture of all four fractions would still be in excess of what could be obtained had the B and C fractions remained unattacked.

A second series of experiments entirely confirmed these results. It must be assumed therefore that the B and C fractions can be converted into alcohol to some slight extent when present in a fermenting solution of fractions D and E, though hardly perhaps to the extent stated by Tanret.

The fermentation experiments referred to above as being difficult to reconcile with the presence in autumn tubers of the Jerusalem artichoke of large percentages of inulin were carried out during November 1922 with samples of juice pressed from freshly-dug tubers and fermented with yeast after dilution and sterilisation in the cold by filtration through Chamberland filters. After inoculation with a yeast this cold sterilised juice was incubated at 35° for 6 days. A yield of alcohol was obtained equal to 47·5 % of the total carbohydrates present. As the percentage of carbohydrates present was found to be equal to that of the fresh tubers it is clear that the inulin fractions B and C must have been present in the tubers and, therefore, must have been almost completely fermented under the conditions of the experiment.

Tanret has shown, and the writers have confirmed, that a partial conversion of the inulin fractions into alcohol may occur when these fractions are present in a vigorously fermenting mixture of other carbohydrates, but this partial conversion is hardly sufficient to explain the ease with which practically the whole of the carbohydrates of the cold sterilised juice was found to be converted.

That bacteria, possessing inulin-inverting enzymes, had developed in the cold sterilised fermenting juice is out of the question, since a careful bacteriological analysis proved the juice to be free from all micro-organisms except the yeast used as inoculant. A further possibility, that the sterilisation of the juice had caused the B and C fractions to become inverted, does not arise in this connection, since no heat had been used for the purpose.

One is almost compelled to assume, therefore, that the cold sterilised juice had contained inulase, which remained sufficiently active after filtration to convert the B and C fractions into fermentable compounds during the 6 days during which the juice remained incubated at 35°.

Attempts made to confirm experimentally the assumption of the presence of inulase in the tubers have not met with very great success. In one experiment a mixture of the B and C fractions in the proportions in which they occur in the autumn tubers was added to juice collected from March tubers. It was thought that this juice would contain considerable quantities of inulase and would be capable of inverting the B and C fractions when allowed to act on them under the conditions used in the experiment with cold sterilised juice. After incubation at 35° for 8 days, however, in the presence of toluene to check the development of micro-organisms, only insignificant quantities of the inulin fractions had disappeared. The actual figures in this experiment were as follows: 90·6 % of the inulin fractions were recovered from the incubated test sample of spring juice containing the B and C fractions by the usual procedure of isolation, as against 93·6 % from the control experiment which had not been incubated. A satisfactory explanation of the observations on the fermentability of the cold sterilised juice obtained from autumn-gathered tubers therefore cannot be given.

Table IX.

Sample	Percentages of inulin fractions (B + C) recoverable from mashes autoclaved for: 6 hours (steaming)	1 hour at 20 lbs.	2 hours at 20 lbs.	3 hours at 20 lbs.	5 hours at 20 lbs.
Spring juice + B and C fractions in the proportions found in autumn tubers	99·9	90·9	66·8	30	11·4

Table X.

Sample	Yield of alcohol calculated on the carbohydrates present obtainable from samples of inulin containing juice after heating in the autoclave at 20 lbs. for: 6 hours (steaming)	1 hour	2 hours	3 hours	5 hours
Sample juice + B and C fractions in the proportions found in autumn tubers	44·3 %	42·6 %	42·6 %	48·4 %	44·7 %

The control experiment consisting of juice from spring tubers without any inulin fraction added gave a yield of 47·3 % alcohol calculated on the carbohydrates present in the mash.

From a practical point of view this question is not of very great importance. It is of greater importance to decide whether the cooking to which the tubers are usually subjected before fermentation is sufficient to render their inulin fractions fermentable, or whether a special acid hydrolysis of the tubers is essential for this purpose.

A number of experiments have been carried out to investigate this point. Tables IX and X contain the results obtained.

The following figures were actually obtained when calculating the results of the above experiment as the percentage of carbohydrates left after the completion of the fermentation (Table XI).

Table XI.

	Amount of carbohydrates left in the mash after fermentation, and calculated as percentage of the initial carbohydrates present				
Sample	Steamed for 6 hours	Cooked for 1 hour at 20 lbs.	Cooked for 2 hours at 20 lbs.	Cooked for 3 hours at 20 lbs.	Cooked for 5 hours at 20 lbs.
Spring juice + B and C fractions added in the proportions found in autumn tubers	48·7 %	11·3 %?	22 %	8 %	6·3 %

As a control, the spring juice without addition of the inulin fractions was fermented, and showed 5·7 % of the original carbohydrates remaining after fermentation.

It will be seen that boiling the mashes at a comparatively low pressure for a considerable time is quite sufficient to convert the inulin fractions of the tubers into fermentable carbohydrates, and that an almost full yield of alcohol may be obtained without a preliminary acid hydrolysis of the mashes.

Having ascertained the nature of the carbohydrates found in the tubers of the Jerusalem artichoke it was thought of interest to establish their relationship to the carbohydrates present in the actual parenchymatous tissues of the unripe stalks—the so-called pith. This tissue contains, on an average, 3 % of soluble carbohydrates which can be extracted with warm water. Subjecting this extract to the process used for isolating the carbohydrates of the tubers the following fractions were collected (Tables XII and XIII):

Table XII.

	Obtained by precipitation			
Fraction	with 55 % alcohol (B)	with 85 % alcohol (C)	with 98 % alcohol (D)	with ether and alcohol (E)
Percentage of total carbohydrates	25·8	4·73	46·27	23·20

The four fractions isolated showed the following properties.

Table XIII.

Fraction	Specific rotation $[\alpha]_D^{20°}$ Before inversion	Specific rotation $[\alpha]_D^{20°}$ After inversion	Reduction of Fehling's solution %	Solubility in water	Appearance
B	−35·4°	−85·6°	1·51	Slightly soluble	Snow-white light powder, tasteless
C	−30·71°	−74·29°	2·41	Slightly more soluble than B	Slightly yellowish sandy powder, slightly sweet
D	−17·91°	−59·39°	12·43	Soluble in all proportions of water	Yellow powder, giving brown solutions, very sweet
E	?	?	?	Soluble in all proportions of water	Light yellow gum giving solutions of the same colour, very sweet

It will be seen that not only are the substances isolated from the pith substantially the same as those isolated from the tubers, but they also appear to occur in the pith in about the same proportions as in the tubers.

All fractions isolated from the pith were tested in the same way as those isolated from the tubers and were found to behave similarly.

Summary.

It has been shown that the autumn-gathered tubers and the unripe pith of the Jerusalem artichoke contain considerable proportions of an insoluble non-fermentable carbohydrate, apparently identical with inulin.

This carbohydrate disappears almost completely from the tubers towards spring and gives way to increased proportions of the more soluble carbohydrates which are already present in the autumn tubers. In addition there would appear to occur towards spring an increase in the dextrorotatory compounds of the tubers. Part of these at least is believed to be sucrose.

Though inulin by itself is unfermentable by yeast the experiments quoted in this report would appear to show that a comparatively mild autoclaving is sufficient to render it fermentable without the use of acid hydrolysis.

The observation of Tanret that inulin may be fermented by yeast when present in a solution of already fermenting carbohydrates has been confirmed, though not to the extent claimed by him.

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LIII. THE ACTIVATION OF CERTAIN OXIDASE PREPARATIONS.

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PART I. ACTIVATION BY PEROXIDASES.

1. *Lactarius* fungi.

Several instances have been noted in the past of the activation by one means or another of a preparation containing some oxidising enzyme. The best-known instance is that described by Bach and Chodat [1902; 1903, 1, 2] of the activation of the oxidase of the fungus *Lactarius vellereus* by peroxidases. The oxidase was obtained by precipitation of the juice of the fungus with three volumes of alcohol. By repeated precipitation of this oxidase in 40 % alcohol, it was separated into two fractions, neither of which showed much oxidising power apart from the other. The one fraction, insoluble in 40 % alcohol, was very strongly activated by peroxidases, and especially by the other fraction of the oxidase, which was soluble in 40 % alcohol. These authors concluded from their observations that the complete oxidase consisted of two enzymes, (1) an "oxygenase" able to give rise in air to a peroxide utilisable by peroxidase, and (2) peroxidase. On this basis they developed their well-known theory of the constitution of the "direct" oxidases.

In their quantitative experiments, Bach and Chodat used pyrogallol as substrate and measured the activity of their preparations by noting the oxygen absorbed. The experiments were carried out without control of hydrogen ion concentration, and no allowance was made for the autoxidation of pyrogallol. Because of this and the advances in our knowledge of oxidases which have taken place since Bach and Chodat published these experiments, certain possibilities arise which must be considered before the explanation given by them can be accepted.

(1) A change in hydrogen ion concentration by addition of peroxidase preparations with a p_H different from that of the pyrogallol-oxygenase solution might of itself cause an increased activity of the oxidase in *Lactarius*.

(2) Hydrogen peroxide might be produced by the action of an enzyme on pyrogallol, the hydrogen peroxide being normally broken down by catalase, but utilisable instead by peroxidase for oxidation of pyrogallol with consequent increase in oxygen absorption.

(3) There might be oxidation by the action of peroxidase and peroxide of substances in the *Lactarius* preparation which inhibit the enzyme acting on pyrogallol, the peroxide being either (*a*) hydrogen peroxide produced by the

action of the enzyme, or (*b*) a peroxide utilisable by peroxidase and produced by autoxidation of some substance in *L. vellereus*. The possibility of this explanation is based on observations by Bach [1909], who found by estimation of unchanged tyrosine that the action of tyrosinase from *Russula delica* on tyrosine was retarded by peroxidase, even, to some extent, in presence of hydrogen peroxide, although the same end-point was approached. He found, however, that if the tyrosinase preparation were old, then peroxidase with hydrogen peroxide caused initial activation. Bach concluded that substances inhibitory to the action of tyrosinase were produced gradually in the preparation on keeping, these substances being oxidisable by peroxidase with hydrogen peroxide.

(4) The presence in peroxidase preparations of something other than peroxidase itself might be responsible for the increased oxygen absorption. A co-enzyme might facilitate the action of a *Lactarius* enzyme on pyrogallol, and if such co-enzyme were deficient in the *Lactarius* preparations but present in the peroxidase preparation added, activation would be produced which would not be due to peroxidase itself.

(5) With regard to the use of pyrogallol as substrate, Bach and Sbarsky [1911] described the formation from pyrogallol of two types of oxidation product, insoluble purpurogallin, and soluble brownish substances which they believed to be condensation products of purpurogallin. The possibility was therefore to be considered of the directing by peroxidase preparations of the oxidation of pyrogallol in such a way as to increase the amount of oxygen absorbed.

(6) Finally, the "oxygenase" of Bach and Chodat might be interpreted as an autoxidisable substance rather than as an enzyme, and without being necessarily a constituent of all oxidases. That "oxygenase" was such an autoxidisable substance was the conclusion reached by Gallagher [1923] in his investigation of potato "oxygenase."

The phenomenon described by Bach and Chodat [1903, 2] has therefore been more fully investigated with regard to these various points.

Enzymes concerned.

Lactarius vellereus not being available, other *Lactarius* fungi were used in this investigation, namely, *L. blennius*, *L. quietus*, *L. rufus*, and *L. turpis*[1]. The action of appropriate preparations from these fungi on pyrogallol was found to be activated by peroxidase from horseradish, in the manner described by Bach and Chodat for the oxidase of *L. vellereus*, but effective peroxidase preparations from these juices were not readily obtained.

These fungi all contained, as likewise described by Chodat [1910] for *L. vellereus*, both tyrosinase and laccase, but in varying proportions. Guaiacol affords a convenient test for laccase, as it is not oxidised by tyrosinase. Quinol, also, is relatively slowly attacked by tyrosinase. *L. turpis* juice caused much less rapid coloration of buffered guaiacol and quinol solutions than of tyrosine

[1] I am indebted to Dr J. Ramsbottom for kind assistance in identifying some of the fungi.

and *p*-cresol, whereas *L. blennius*, *L. quietus*, and *L. rufus* juices all acted very rapidly on guaiacol and quinol relatively to their action on tyrosine and *p*-cresol. The tyrosinase in these juices could be freed from laccase by alcohol precipitation. A preparation obtained from *L. blennius* juice by two precipitations in 40 % alcohol was found to behave towards phenols as does crude tyrosinase from mealworms, having no action on guaiacol with or without addition of hydrogen peroxide. A similar preparation was obtained from *L. turpis* by 40 % alcohol, and from *L. rufus* in the fraction from 65–80 % alcohol. Tyrosinase appears to be less definitely separated from the juices by alcohol precipitation than is laccase. Fractions obtained from 40–60 % alcohol acted strongly on guaiacol, but were not free from tyrosinase. The two enzymes appear to possess differing susceptibilities to cyanide. The action of *L. quietus* juice on tyrosine was found to be 95 % inhibited by *M*/500 KCN, that of *L. rufus* juice 97 %; whereas Wieland and Sutter [1928] stated that the action on quinol of the enzyme from *L. vellereus* investigated by them was only 67 % inhibited by *M*/500 KCN.

After being heated to 90° for 20 minutes none of the *Lactarius* juices used in the present investigation had any appreciable action on either tyrosine or guaiacol; the thermostable catalyst described by Wieland and Fischer [1926] was therefore not present in any appreciable quantity in any of them. The 40 % alcoholic precipitates all contained some tyrosinase and catalase, and some contained laccase. The "peroxidase" fractions of the fungi prepared as described below all contained tyrosinase, and some contained laccase; the guaiacol-hydrogen peroxide test for peroxidase was positive only with *L. blennius* and *L. turpis* "peroxidase."

Experimental procedure.

Juices of *L. blennius*, *L. quietus*, and *L. turpis* were kept in the ice-chest at a slightly alkaline reaction, and acidified before precipitation to imitate as nearly as possible the reaction of the fresh juices. Some *L. rufus* juice was precipitated fresh, and the remainder was kept in the same way as the other juices. "Oxygenase" fractions were obtained by addition of alcohol to the extent of 40 %, the precipitate being centrifuged, dissolved in slightly alkaline water (residue discarded), acidified, and reprecipitated in 40 % alcohol, as many times as possible with the material available. The first 40 % alcoholic liquid after removal of the precipitate was concentrated *in vacuo*, then alcohol was added to bring it to 65 %, the precipitate centrifuged, and alcohol added to the liquid to bring it to 85 %; this last precipitate was dissolved in water and reprecipitated between the limits about 65–85 % alcohol, as many times as possible, the final solution constituting the "*Lactarius* peroxidase" fraction. Peroxidase from horseradish was prepared by the method described by Bach and Chodat [1903, 1], and, unless otherwise stated, such a preparation is what is meant by the term peroxidase.

The action of "oxygenase" fractions on pyrogallol, and the effect thereon of addition of peroxidases, was investigated by the use of microrespirometers

to measure the rate of oxygen absorption. Throughout the experiments the respirometers were shaken in a water-bath at room temperature, in which they were equilibrated for at least five minutes before the shutting of the taps. Corresponding experiments were shaken side by side, and in such experiments identical buffer solution was used. The following mixtures were placed in the bottles.

Respirometer I. Left: 1 cc. 1·2 % pyrogallol in phosphate buffer p_H 6·0; 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Respirometer II. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase from horseradish or *Lactarius*; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase; buffer to 3 cc. *Respirometer III. Left:* 1 cc. 1·2 % pyrogallol; buffer to 3 cc. *Right:* 3 cc. buffer. *Respirometer IV. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. peroxidase; buffer to 3 cc. *Right:* 0·5 cc. peroxidase; buffer to 3 cc. To each mixture a little phenylurethane or thymol was added as antiseptic, and in the small tube of each bottle was placed excess of strong potash to absorb carbon dioxide. The use of pyrogallol solutions stronger than 1·2 % (2·5 and 5 %) did not increase the rate of oxygen absorption.

Any departures from the above arrangements are mentioned in the text. In calculation of the activation of the "oxygenase" fraction by peroxidases, allowance was made for the effect of the peroxidase preparation used on the autoxidation of pyrogallol, as follows:

$$\text{activation ratio} = \frac{O_2 \text{ absorbed in II} - O_2 \text{ absorbed in IV}}{O_2 \text{ absorbed in I} - O_2 \text{ absorbed in III}}.$$

Ratios above unity indicate activation and below unity inhibition.

Control. The presence of substrate was necessary for the phenomenon of activation to occur. Using *L. quietus* "oxygenase" fraction, in absence of pyrogallol, Respirometer I absorbed over the week-end 19·8 mm.^{3} O_2, II 25·7 mm.^{3} O_2, IV 6·4 mm.^{3} O_2.

Experimental results.

Using "oxygenase" fractions from the fungi named, and peroxidase (*a*) from horseradish, (*b*) from the fungus itself, the following results were obtained:

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers I	II	III	IV	Activation ratio
			(*a*) Horseradish peroxidase				
L. blennius	2	6	32·6	42·5	19	15	2·02
L. quietus (1)	4	6	101	288	18·5	11·7	3·33
„ (2)	4	1	313	356	10·5	8·7	1·15
		3	533	667	21	15	1·27
		6	783	1110	29	22·5	1·44
L. rufus	3	6	371	401	30	23	1·11
L. turpis	2	6	55	58	38	33	1·47
			(*b*) Fungus peroxidase				
L. blennius	2	6	32·6	49·7	19	27·3	1·65
L. quietus	4	6	101	111·5	18·5	29	1
L. rufus	1	6	384	454	30	113	0·96
L. turpis	1	4	44	292	28	268	1·50

In no instances were activations observed as great as those described by Bach and Chodat. This may be due to the phenomenon being better shown by *L. vellereus* than by the varieties of *Lactarius* used in the above experiments.

Most of the horseradish peroxidase preparations were found to retard the autoxidation of pyrogallol, and either to retard the action of the "oxygenase" fraction of *Lactarius* juices on pyrogallol in its early stages, or to produce less activation at the beginning of the experiment than later: figures quoted for *L. quietus* (2) in the first table show this increase in activation ratio with time. The following experiments, in which diluted, unprecipitated juice was used in place of "oxygenase" fraction, and the p_H buffered at 6·5, also show increase in activation with time.

Fungus	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
L. rufus	½	13	10	5	3	0·87
	1	20	21	9	7	1·27
	2	35	40	15	12	1·40
	4	64	83	25	20	1·62
	10	154	224	45	40	1·69
L. quietus	1	120	99	9	11	0·79
	2	206	199	15	17	0·95
	3	287	297	20	23	1·02
	6	475	551	33	37	1·16
	10	629	794	45	53	1·27

The various points raised in connection with the observed activation phenomenon will now be considered in turn, and experiments bearing on them described.

(1) *Possibility of change in hydrogen ion concentration.* Since the solutions were buffered, this effect is minimised. Some of the horseradish peroxidase preparations, which consisted of alcoholic precipitates dried *in vacuo* and dissolved in distilled water, were found to be acid, and the buffering was not able to prevent slight decrease in p_H on addition of such peroxidases. Since Wieland and Sutter [1928] found the optimum p_H for the action of the enzyme from *L. vellereus* investigated by them to be 4·6, using quinol as substrate, a decrease in p_H might of itself activate the *Lactarius* oxidase. The effect of change in p_H on the oxygen uptake accompanying the action of *L. rufus* juice on pyrogallol was therefore examined.

p_H	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	III	I–III
6·0	8	74	27	47
6·5	8	142	34	108
7·0	8	279	242	37

It is shown in the middle column that decrease in p_H did not increase the oxygen taken up by the autoxidation of pyrogallol, and in the last column that, when allowance was made for the autoxidation of pyrogallol, decrease in p_H from 6·5 did not increase the oxygen taken up by the action of enzymes in the juice on pyrogallol. Hence, change in hydrogen ion concentration from a higher to a lower p_H cannot explain the phenomenon of activation.

(2) *Possibility of hydrogen peroxide production* by the action of an enzyme or enzymes in *Lactarius* preparations on pyrogallol. Some catalase was present in all *Lactarius* preparations, so that if hydrogen peroxide were produced and normally broken down by catalase, but on addition of peroxidase utilised for oxidation instead, then there would be an increase in oxygen absorption. This increase, however, would depend on the rate of production of hydrogen peroxide, and in no way could exceed a doubling of the rate. Higher rates than this are here recorded, and the activations observed by Bach and Chodat were even greater. Hydrogen peroxide production in this way, therefore, cannot provide a complete explanation of the phenomenon. The possibility is not precluded that it does take place, and in conjunction with (3), the oxidation of inhibitory substances in *Lactarius* might explain the phenomena.

The work of Wieland and Sutter [1928] furnishes no evidence of the production of hydrogen peroxide by enzymes in *L. vellereus* other than the thermostable catalyst described by Wieland and Fischer [1926]. The former investigators failed to detect any hydrogen peroxide production by their enzyme preparation from *L. vellereus* (apparently a laccase) under conditions in which its production by the Wieland and Fischer catalyst was readily demonstrated.

(3) *Possibility of oxidation of inhibitory substances.* Since the activation ratio increased with time, it seems probable that inhibitory substances either in the *Lactarius* or in the peroxidase preparations added were being oxidised on addition of peroxidase. In this connection the work of Szent-Györgyi [1928] on an inhibitory hexuronic acid oxidisable by hydrogen peroxide in presence of peroxidase and a phenol is of interest.

A consideration against the presence of inhibitory substances in the *Lactarius* preparations is the control experiment described earlier in this paper, which shows that there cannot be present both inhibitory substances oxidisable by peroxidase and peroxide, and substances autoxidisable with production of peroxide. If present along with such autoxidisable substances, they would in any event be oxidised even without addition of peroxidase, by means of peroxidase in the juices. If, however, hydrogen peroxide were derived from the action of an enzyme on pyrogallol, the presence of inhibitory substances in the *Lactarius* preparations, oxidisable by peroxidase + peroxide, might explain the phenomena, since then much greater activation than a doubling in the rate would be possible. They cannot be of the type described by Bach [1909], since, on keeping the *Lactarius* juices under slightly alkaline conditions, their activation by peroxidase did not increase, but eventually disappeared before either tyrosinase or laccase had lost all their activity, tyrosinase being most persistent.

(4) *Possibility of effect not being due to peroxidase itself.* The activation by peroxidase of the action on pyrogallol here discussed appears to be of an entirely different type from the activation of the action of tyrosinase on a monohydric phenol by boiled tyrosinase preparations, or by *o*-dihydric phenols, discussed later in this paper.

In some qualitative experiments on the liberation of iodine from potassium iodide and the blueing of guaiacum by "oxygenase" with peroxidase, Bach and Chodat [1902] record a control with boiled peroxidase preparation, which did not cause activation. In the present work it was shown that the phenomenon under investigation was likewise not brought about by boiled peroxidase preparation, since, when peroxidase which had been boiled for some time to destroy its activity was used, no activation of the action of *L. rufus* juice was observed.

Duration of experiment in hours	p_H	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
9	6·5	132	139	37	46	0·98

Use of an amino-acid, glycine, in place of peroxidase preparation did not cause activation of the action of the juice. (Amino-acids are not oxidised by the oxidation products of pyrogallol produced by mealworm tyrosinase. *Respirometer. Left:* 1 cc. 0·2 % pyrogallol in phosphate buffer, p_H 6·0; 1 cc. 0·45 % glycine in buffer; 0·5 cc. mealworm preparation; buffer to 3 cc. *Right:* 1 cc. 0·2 % pyrogallol; 0·5 cc. mealworm preparation; buffer to 3 cc. No change in levels occurred. Tyrosine in place of pyrogallol yielded a similar result, as also did quinol.)

L. rufus juice which had been dialysed to remove any hypothetical dialysable co-enzyme was not more activated by peroxidase than juice which had not been dialysed.

Description of preparation	p_H	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
Undialysed	6·5	8	134	183	20	43	1·23
Dialysed	6·5	8	47	65	29	44	1·17

There was, therefore, no dialysable co-enzyme in the juice, which was supplied by the peroxidase preparations.

(5) *Possibility of change in nature of oxidation products.* Unless excess substrate be present, the rate of action cannot be assumed to be a measure of the activity of the enzyme. When dilute (0·2 %) pyrogallol solutions were used no difference in rates was detectable with or without addition of peroxidase. The oxygen absorption, however, with or without addition of peroxidase, exceeded in a few hours the total oxygen absorption produced by the action of another enzyme preparation, tyrosinase from mealworms, on the quantity of pyrogallol used.

Required for 1 cc. 0·2 % pyrogallol:

calculated value for the production of purpurogallin	266	mm.³ O_2
by action of tyrosinase from mealworms; final figure	311	,,
,, *L. quietus* "oxygenase" fraction; 1 day	448	,,
,, ,, ,, ,, + peroxidase; 1 day	398	,,

Since the excessive oxygen absorption took place with or without addition of peroxidase, however, it cannot account for the phenomenon of activation[1].

When 2 cc. saturated tyrosine in phosphate buffer p_H 6·0 was used as substrate in place of pyrogallol, activation by peroxidase was still observed, showing that the phenomenon of activation did not depend on choice of substrate.

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	Activation ratio
L. quietus	4	1	279	267	0·96
		4	403	498	1·24

Wieland and Sutter [1928], using quinol as substrate, tested the action of peroxidase from horseradish on the oxidase preparation obtained by them from *L. vellereus*. The duration of their experiment was 30 min. They found that 4·96 cc. O_2 were absorbed without, and 5·60 cc. O_2 with, addition of peroxidase. These figures they considered scarcely to exceed their experimental error.

(6) *Possibility of the presence of autoxidisable substance in* Lactarius. This appears to be the most likely explanation of the phenomena observed by Bach and Chodat.

Juices which had been exposed to air were, when afterwards tested on pyrogallol, more readily activated by peroxidase than juices which had been kept *in vacuo*, the initial retardation on addition of peroxidase being counterbalanced sooner by the activating action. The following experiments were carried out with *L. quietus* juice. One sample of juice was exposed to air for about one day while another sample stood in an evacuated desiccator. The activation by peroxidase of the action of each sample on pyrogallol was then determined. 1 cc. was used of a peroxidase preparation which had initially a slightly retarding action, and it was only after a number of hours—given in column A of the following table—that the oxygen absorption with peroxidase became equal to that without peroxidase, the oxygen absorption at which this occurred being given in mm.³ in column B. Beyond this point activation set in. It is seen that activation set in more readily when the fungus juice had previously been exposed to air than when it had been *in vacuo*. A 1 and B 1 denote the results obtained with a sample previously exposed to air, and A 2 and B 2 the corresponding results with a sample previously kept *in vacuo*. The whole experiment was repeated three times.

Experiment	A 1 hrs.	B 1 mm.³	A 2 hrs.	B 2 mm.³
1	2½	288	4½	507
2	2	200	4	300
3	5	209	11½	331

A similar effect was observed with the *L. rufus* "oxygenase" fraction. The initial retardation on addition of peroxidase was less when the "oxygenase" preparation had stood for one day than when it was tested immediately after

[1] It appeared to be due to the oxidation of pyrogallol by *Lactarius* giving rise to products different from those produced by the oxidation of pyrogallol by tyrosinase from mealworms.

preparation. It was usually observed also that the initial retardation was slightly greater when 1 cc. peroxidase was used (II b) in place of 0·5 cc. (II a).

Description of "oxygenase" fraction	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers		
		I	II a	II b
Fresh	1	92	75	—
	2	162	152	—
One day old	1	97	91	89
	2	163	168	162

These results are consistent with the presence in the juices of a substance which on autoxidation produces a peroxide, and consequently increases the action on pyrogallol when peroxidase is added. There appear also to be inhibitory substances in the peroxidase preparations, which cause initial retardation, and are oxidised away by peroxide with peroxidase.

It follows from the consideration of the experiments so far described that the most likely alternative explanation to the presence of an autoxidisable substance in *Lactarius* juices is the production of hydrogen peroxide by the action on pyrogallol of an enzyme in *Lactarius* precipitated by 40 % alcohol, and the oxidation by peroxidase and this hydrogen peroxide of a further amount of pyrogallol, and also of inhibitory substances in *Lactarius* juices. If this had been the complete explanation, then previous exposure to air of the juices would have been expected to have either no influence on the effect of addition of peroxidase, or, if the inhibitory substances became oxidised in air, to diminish rather than accentuate the observed effect on addition of peroxidase. Since accentuation was observed, the effect was probably due to the alternative explanation, namely, the presence of autoxidisable substance. Although this is evidence of the presence of autoxidisable substance, the production of hydrogen peroxide by the action of an enzyme is not excluded as a partial explanation of the phenomenon.

Further support of the above view was obtained. If by any means the oxidising activity of the juices could be diminished without a corresponding diminution in the degree of activation by peroxidase, then the activation would appear to be a phenomenon not entirely dependent on the action of the enzyme which oxidises pyrogallol. Cyanide was found to affect the action of the enzyme in the juice on pyrogallol much more than it did the process responsible for activation, thus causing a relatively large increase in activation.

Exp. 1. A sample of *L. quietus* juice was tested without cyanide, using 1 cc. peroxidase, and working at p_H 6·5.

	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Exp. 1	7	675	766	36	29	1·15
Exp. 2	7	156	335	—	—	2·15

Exp. 2. 0·5 cc. $3M/250$ neutralised KCN in buffer p_H 6·5 was added to each bottle of each respirometer, bringing the cyanide concentration to $M/500$, and using otherwise the same materials as in Exp. 1. No alteration in p_H of the

liquids during the experiment was detectable. *M*/500 KCN practically stopped the autoxidation of pyrogallol.

Retardation by cyanide of the action of the juice on pyrogallol was as follows:

Duration of experiment in hours	mm.³ O_2 absorbed in respirometers		Percentage retardation
	Without KCN	*M*/500 KCN	
6	634	132	79

The phenomenon of activation disappeared when the juice had been heated. *L. quietus* juice, which had been heated at 90° for half an hour, gave the following results:

Treatment of juice	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
			Activation			
Heated	7	64	50	29	29	0·60
			Activity of juice alone			
Unheated	6	634	—	25	—	—
Heated	6	55	—	25	—	—

The loss of activity of the juice itself on heating was 95 %.

Discussion.

The investigation which has been carried out shows that the phenomenon discovered by Bach and Chodat [1903, 2] of the activation by peroxidase of the action on pyrogallol of *Lactarius vellereus* oxidase takes place when certain other *Lactarius* fungi are used. In recent years it has been considered likely that the explanation of the observations which led Bach and Chodat to the formulation of their theory of the constitution of "direct" oxidases was the presence of substances autoxidisable to peroxides. This explanation has been found to be applicable in the present instance. There are in the fungi, in all probability, substances autoxidisable to peroxides utilisable by peroxidase; the addition of peroxidase therefore causes oxidation of further pyrogallol, an effect which is enhanced by removing peroxidase already present in the juices by fractional precipitation with alcohol. It is possible that, besides this, one of the oxidases in the juice may produce hydrogen peroxide when acting on pyrogallol, and may therefore be able to form with peroxidase a system analogous to that discovered in milk by Thurlow [1925], thus providing a partial explanation of the phenomenon of activation by peroxidase. As already pointed out, no thermolabile oxidase in *Lactarius* has been shown to have this function, but it is possible that one may be present able to act in this way, and it is intended to investigate the matter when further material is available. The presence of autoxidisable substance precludes the co-existence in *Lactarius* juices of inhibitory substances oxidisable by peroxidase and peroxide, without which the production of hydrogen peroxide by the action of an enzyme on pyrogallol cannot furnish a complete explanation. The increase in activation ratio with time is probably due to the presence of such inhibitory substances in peroxidase prepared by the method used. The presence of these inhibitory

substances may account for the difference in activating power of peroxidases from different sources observed by Bach and Chodat.

Evidence was not obtained that the oxidases in *Lactarius* juices are separable solely into enzyme-like peroxide and peroxidase, the original view of Bach and Chodat [1903, 2]. A 60–80 % alcohol precipitate from *L. turpis* juice, which was allowed to stand in the ice-chest in contact with 80 % alcohol for 10 days, lost its activity on tyrosine and on guaiacol, although acting slightly on guaiacol after addition of hydrogen peroxide. Since neither the "oxygenase" nor "peroxidase" fraction of *L. vellereus* oxidase obtained by Bach and Chodat had much oxidising power apart from each other, it seems possible that by repeated alcoholic precipitation Bach and Chodat largely destroyed tyrosinase and laccase in both fractions, and separated from one another autoxidisable substance and peroxidase. The view that tyrosinase and laccase are separable into peroxidase and autoxidisable substance does not appear tenable. It seems necessary at present to regard tyrosinase, laccase, and peroxidase with autoxidisable substance as separate distinct systems.

2. Other oxidase preparations.

A. Potatoes. Potatoes were minced and pressed, and the juice was centrifuged; the liquid was precipitated once in 40 % alcohol and the precipitate dissolved as described for *Lactarius* juices. The potato preparations contained tyrosinase, laccase, and catalase. The action of such potato preparations on pyrogallol at p_H 6·5 was not activated by peroxidase, neither was new potato juice unprecipitated by alcohol activated by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Old potatoes	11	138	161	31	46	1·07
Old potatoes	8	132	131	22	54	0·70
New potatoes	11	112	102	31	76	0·32

These results are unexpected in view of the work of Gallagher [1923] on an autoxidisable substance in potatoes. The peroxidase preparations were all neutral; the presence in them of true inhibitory substances is therefore probable. Dialysis of the potato preparations did not result in activation by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Old potatoes	11	101	68	31	46	0·31
New potatoes	11	137	134	31	76	0·55

B. Mealworms. Owing to the absence of laccase, a preparation from mealworms affords convenient material for investigation of the action of peroxidase on the activity of tyrosinase. The preparation contains much catalase, but appears to contain no peroxidase, since it does not act on guaiacol even after addition of hydrogen peroxide, under which conditions a trace of added peroxidase causes immediate coloration of guaiacol. Using the mealworm preparation, prepared according to Raper [1926], filtered, the results (1) were

obtained; dialysed and centrifuged, the results (2). A similar preparation several months old, filtered, was activated (3). The results (4) were obtained by using 2 cc. saturated tyrosine in buffer as substrate in place of pyrogallol, with the mealworm preparation used in (1). The p_H was 6·5, and all peroxidase preparations were neutral.

Description of experiment	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
(1)	10	314	285	23	26	0·89
(2)	7	373	319	31	28	0·85
(3)	10	55	99	23	26	2·28
(4)	8	210	143	—	—	0·68

Thus, only the old preparation was activated by peroxidase. This supports the observations made on *Lactarius* preparations, which indicate that the presence of tyrosinase in the "oxygenase" fractions was not responsible for the activation phenomenon. Since the peroxidase preparations even when neutral had an inhibitory action, the presence in them of inhibitory substances is probable.

The observation recorded by Bach [1909], of the action of peroxidase on tyrosinase, was confirmed qualitatively, using a preparation from mealworms obtained by preliminary extraction with 60–80 % saturated ammonium sulphate, followed by extraction with alkaline water. The darkening of tyrosine by tyrosinase was initially impeded by peroxidase, in presence or absence of hydrogen peroxide, but more in its absence. After one day, all four mixtures were equally dark.

PART II. ACTIVATION OF TYROSINASE.

1. Activation by *o*-dihydric phenols.

It was shown by Raper [1926] that the oxidation of tyrosine by tyrosinase from mealworms was initially activated by 3 : 4-dihydroxyphenylalanine. During the course of the present research, the oxygen absorption accompanying the action of tyrosinase on tyrosine was measured[1]. It was found that the absorption did not start at once, but gradually increased to a constant rate, indicating that the reaction is autocatalytic. This is in agreement with the "catechol" theory of Onslow [1923], as pointed out by McCance [1925]. The addition of dihydroxyphenylalanine or of catechol caused activation by eliminating the initial lag. That this is the interpretation of the phenomenon appears from a recalculation of Raper's figures as rates.

Times (minutes)	0	65	125	205
	Tyrosine oxidised per 20 cc. (mg.)			
Without dihydroxyphenylalanine	0	1·15	2·44	3·54
With dihydroxyphenylalanine	0	1·49	2·54	3·47
Whence, rates per 5 minutes—				
Without dihydroxyphenylalanine	0·088	0·107	0·07	—
With dihydroxyphenylalanine	0·114	0·09	0·06	—

The enzyme used by Raper had not been dialysed.

[1] It amounts to a little over five atoms per molecule of tyrosine; the action on dihydroxyphenylalanine is accompanied by an absorption of a little over four atoms per molecule.

In the present experiments, the following mixtures were placed in respirometers. I. *Left:* 0·4 mg. tyrosine; 0·5 cc. tyrosinase from mealworms; buffer to 3 cc. *Right:* 0·5 cc. tyrosinase; buffer to 3 cc. II. *Left:* 0·4 mg. tyrosine; 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. *Right:* 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. The p_H was 6·0. The oxygen absorbed in mm.^{3} was as follows:

Rates per 20 min.
I 2; 12; 14; 11; 9; 7; 5.
II 6; 6; 6; 7; 7; 7; 6.

1 cc. saturated tyrosine in buffer was then used as substrate and 0·1 mg. dihydroxyphenylalanine as activator, p_H 7·0:

Rates per 15 min.
I 0; 5; 20; 27; 22; 23; 17; 13.
II 25; 26; 20; 21; 15; 13; 10; 6.

There was no initial lag when, in place of tyrosine, dihydroxyphenylalanine was used as substrate. Using 1 mg. dihydroxyphenylalanine at p_H 6·0:

Rates per 7 min.
I 12; 10; 9; 8; 8.

The enzyme used in the above experiments had been dialysed.

Undiluted tyrosinase preparation acted too rapidly on *p*-cresol for the initial lag to be obvious, but on dilution of the enzyme it became apparent. Using 2 mg. *p*-cresol as substrate, and 0·05 mg. catechol as activator, at p_H 7·0:

Rates per 10 min.
I 3; 9; 10; 13; 13; 12; 11.
II 10; 13; 12; 12; 12; 12; 11.

There was no initial lag when catechol was used as substrate.

Using 2 mg. catechol, at p_H 7·0:

Rates per 5 min.
I 10; 9; 8; 10; 7; 6; 6.

Using enzyme still further diluted:

Rates per 5 min.
I 3·6; 2·9; 3·9; 3·3; 4·2; 2·6; 3·6; 2·9.

The enzyme used in the above experiments had not been dialysed.

In the following experiments the enzyme had been dialysed.

Using 2 mg. *p*-cresol at p_H 7·0:

Rates per hour
I 0; 3; 15.

Using 2 mg. catechol at p_H 7·0:

Rates per 15 min.
I 5; 5; 4.

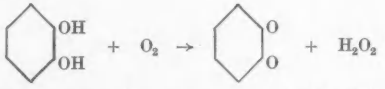
A similar effect was observed when a *L. turpis* "oxygenase" fraction was used instead of mealworm tyrosinase. Using 2 mg. *p*-cresol as substrate, and 0·06 mg. catechol as activator, at p_H 7·0:

Rates per hour
I 0; 0; 0; 19; 26.
II 5; 11; 22; 22; 26.

Discussion.

The phenomena here described have a significant bearing on the mode of action of tyrosinase. Since the action on the monohydric phenols tyrosine and *p*-cresol is autocatalytic, and the lag is eliminated by addition of an *o*-dihydric phenol, it appears as if some product of action on an *o*-dihydric phenol enabled the enzyme to act on the monohydric phenol. Since dialysis of the enzyme preparation did not destroy its action on the monohydric phenol, the necessary product appears not to be dialysable. It is quite likely, however, that in some instances the requisite *o*-dihydric phenol may be present as an impurity in the monohydric phenol, a possibility included by Onslow [1923]. Some old samples behaved towards peroxidase and hydrogen peroxide as if they contained some corresponding *o*-dihydric phenol, and such samples showed no initial lag when tyrosinase acted on them. This possibility is under further investigation, although it cannot furnish a complete explanation, as follows from the work of Onslow and Robinson [1928] described below.

The production of hydrogen peroxide by the action of tyrosinase has not so far been demonstrated, and if the preparation from *L. vellereus* used by Wieland and Sutter [1928] contained tyrosinase, then the production of hydrogen peroxide by tyrosinase is unlikely. The production of *ortho*-quinones has been demonstrated [Happold and Raper, 1925; Pugh and Raper, 1927]. If, nevertheless, the reaction



expresses what occurs when tyrosinase acts on an *o*-dihydric phenol, in accordance with the suggestion of Onslow and Robinson [1926], then both *ortho*-quinone and hydrogen peroxide are possible activators of the action on a monohydric phenol. Onslow and Robinson [1928] believe the effective substance to be an *ortho*-quinone. Using an enzyme obtained from the potato by preliminary treatment with alcohol, followed by aqueous extraction of the enzyme, they obtained, by means of treatment with charcoal, preparations which acted strongly on catechol, but scarcely at all on *p*-cresol or tyrosine. Addition of a trace of catechol caused action on *p*-cresol, and slight action on tyrosine. They concluded from their experiments that the oxidation of the monohydric phenols is a secondary phenomenon depending on the presence of an *ortho*-quinone and not directly on the action of the enzyme. They assumed that alcohol did not extract the *ortho*-quinone from plant tissue, and that it was an *ortho*-quinone which was removed by charcoal.

An *ortho*-quinone has not been shown to convert a monohydric to an *o*-dihydric phenol. Also it seems unlikely that an *ortho*-quinone is the effective substance, because dihydroxyphenylalanine will serve as activator, although the *ortho*-quinone formed from it is not "free" (as shown by its inability to oxidise an external amino-acid), but condenses at once with another part of its own molecule [Raper, 1927]. The following considerations indicate that

neither *ortho*-quinone nor hydrogen peroxide is able to bring about the conversion of a monohydric to an *o*-dihydric phenol at the requisite rate (the rate of a series of reactions being determined by the rate of the slowest), unless possibly it is catalysed in some way. Peroxidase with hydrogen peroxide, as well as tyrosinase, produces *ortho*-quinones from *o*-dihydric phenols such as catechol [Pugh and Raper, 1927], yet peroxidase with hydrogen peroxide does not act on tyrosine, even in presence of a trace of added catechol, although peroxidase with hydrogen peroxide is known to act rapidly on dihydroxyphenylalanine, the *o*-dihydric phenol produced from tyrosine by tyrosinase [Raper, 1926], to give products indistinguishable from those produced by tyrosinase. Neither does peroxidase with hydrogen peroxide act on phenol. It follows, since peroxidase with hydrogen peroxide is able to carry out all further stages in the reactions, that neither hydrogen peroxide nor *ortho*-quinone is able to convert monohydric to *o*-dihydric phenol, unless the change is catalysed by something specifically present in tyrosinase preparations. Until enzymes catalysing action on monohydric and *o*-dihydric phenols have been shown to be distinct from one another, the name "tyrosinase" must be applied to both, since action on tyrosine by means of a co-enzyme is a distinctive property of the enzyme.

The facts may possibly indicate that the conversion of monohydric to *o*-dihydric phenol is brought about by hydrogen peroxide (Dakin's reaction), catalysed by tyrosinase, possibly by way of an oxidised form of the enzyme; but further work is required before the mechanism responsible for action on a monohydric phenol can be known.

2. Activation by boiled preparations.

The observation was made by Haehn [1919], and confirmed by Raper and Wormall [1923], that the action of tyrosinase from potatoes on tyrosine was activated by boiled potato juice. This has been confirmed for tyrosinase prepared from mealworms.

Recalculation of Raper and Wormall's figures in terms of rates shows that the phenomenon consists in a tendency to elimination of initial lag.

	Tyrosine present in g. per 330 cc.				
Ordinary potato juice	0·1427	0·1269	0·1062	0·0905	0·0321
Potato juice + boiled potato juice	0·1442	0·1185	0·0980	0·0804	0·0287
Time (min.)	0	130	220	360	1380

Whence rates per 10 min. (mg.)—

Without boiled juice	1·21; 2·30; 1·12; 0·57.
With boiled juice	1·98; 2·28; 1·26; 0·51.

Using the arrangement of respirometers described for activation by *o*-dihydric phenols, 1 cc. saturated tyrosine as substrate, and 1 cc. boiled mealworm preparation as activator, at p_H 7·0:

	Rates per 15 min.
I	0; 5; 20; 27; 22; 23; 17; 13.
II	25; 30; 23; 21; 14; 12; 7; 7.

The boiled preparation had been dialysed before boiling.

That activation by boiled preparations is related to the activation by *o*-dihydric phenols does not appear unlikely.

Summary.

1. The phenomenon discovered by Bach and Chodat of the apparent activation by peroxidases of a constituent of the oxidase of the fungus *Lactarius vellereus* separable from it by 40 % alcohol is considered.

2. Using peroxidase from horseradish, the phenomenon has been confirmed for some other *Lactarius* fungi, and certain alternative explanations to that put forward by Bach and Chodat are discussed.

3. The most probable interpretation of the phenomenon is the presence in *Lactarius* fungi of an autoxidisable substance. The possible presence also of some enzyme able to produce hydrogen peroxide by dehydrogenation of the substrate may contribute to the phenomenon. No evidence was obtained of the dual constitution ascribed by Bach and Chodat to oxidases in general.

4. The effect of peroxidase from horseradish on the action of potato juice and of tyrosinase from mealworms is described.

5. The action of tyrosinase on tyrosine and on *p*-cresol is shown to be autocatalytic, the lag being eliminated by addition of a small amount of catechol or of dihydroxyphenylalanine.

6. The suggestion put forward by Onslow and Robinson [1928], that an *ortho*-quinone is responsible for initiation of action on a monohydric phenol, is discussed.

7. The elimination of lag by boiled tyrosinase preparations is demonstrated.

I wish to express my thanks to Professor H. S. Raper, under whose direction this work was carried out, for his kind criticism and help throughout the investigation.

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LIV. SOME ENZYMES IN *B. COLI COMMUNIS* WHICH ACT ON FUMARIC ACID.

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It was shown by Quastel and Woolf [1926] that when fumaric acid and ammonia are incubated with a suspension of *B. coli communis* at p_H 7·4 in presence of an "inhibitor," such as 2 % propyl alcohol, there is a disappearance of free ammonia from the solution. Similarly, when *l*-aspartic acid is incubated under the same conditions, it is partially deaminated, and the time-course and final values of the ammonia uptake or output correspond with those required for the reversible reaction

$$COOH.CH=CH.COOH + NH_3 \rightleftharpoons COOH.CH_2.CH(NH_2).COOH$$

where the molecular equilibrium constant $K = \frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}$ has a value of about 0·04. In the absence of "inhibitors," there is also, under anaerobic conditions, an irreversible complete deamination of the aspartic acid, with production of succinic acid. It was shown by Cook and Woolf [1928] that the mechanism responsible for this reductive deamination is independent of the enzyme governing the aspartic-fumaric equilibrium, since it is present in strict aerobes and strict anaerobes, which do not bring about the latter reaction. When equilibrium has been attained, both aspartic acid and fumaric acid can be isolated, but, while the yields of aspartic acid agree satisfactorily with the theoretical, the yields of fumaric acid are invariably very low.

The presence in animal tissues of an enzyme capable of forming malic acid from fumaric acid was shown by Batelli and Stern [1911], who named it "fumarase." Einbeck [1919] reported that the reaction stopped when about three-quarters of the fumaric acid had been converted, and Dakin [1922] confirmed this and proved that the malic acid produced was exclusively the *laevo*-form. Clutterbuck [1927] followed polarimetrically the production of *l*-malic acid from fumaric acid in presence of muscle, and found that the reaction followed a linear course until the equilibrium was nearly reached, when it slowed down. Alwall [1929] completed the proof that a true chemical equilibrium was involved by showing that the same final state was reached when muscle tissue was allowed to act on *l*-malic acid.

The presence of fumarase in *B. coli* was reported by Quastel and Whetham [1924]. It is obvious that if this enzyme were present in the bacillus in any

considerable quantity it would interfere with the aspartic-fumaric equilibrium, giving a complex malic-fumaric-aspartic equilibrium as the final state. Quastel and Whetham, however, found that when fumaric acid was incubated anaerobically with relatively large concentrations of organisms, only minute quantities of malic acid could be detected. They therefore concluded that there was only a small quantity of fumarase in *B. coli*, and in the previous work on the aspartic-fumaric equilibrium this conclusion was accepted, and it was taken for granted that the production of malic acid was so slow as not to interfere appreciably with the main reaction. It will be shown in this paper that the interpretation given by Quastel and Whetham of their results was erroneous, though justified by the facts known at the time they did their work; that, in fact, *B. coli* shows a very high fumarase activity; and that the equilibrium constant previously reported for the aspartic-fumaric equilibrium is really that of the complex malic-fumaric-aspartic equilibrium. It will also be shown that malic acid itself does not take up ammonia, that the addition of water and of ammonia to fumaric acid is due to two distinct enzymes, and that it is possible to eliminate the fumaric-malic reaction and so obtain the true constant of the equilibrium between aspartic acid, fumaric acid and ammonia. The bearing of these results on the "active centre" hypothesis of Quastel and Wooldridge will be discussed.

Experimental.

All the work described was done with suspensions of "resting" *B. coli communis*. The organism was grown, either in Roux bottles containing 150 cc. of Cole and Onslow's tryptic broth, or on the surface of tryptic broth agar in Petri dishes. There was no apparent difference in the behaviour of the organisms obtained by the two methods. In each case, the nutrient medium was inoculated from an 18 hours old broth culture, and incubated at 37° for 2 days. When agar plates were used, the growth was washed off with normal saline and centrifuged; when broth was used, this was centrifuged from the organisms. The deposit of *B. coli* was then washed three times by centrifuging in normal saline, and finally suspended in saline and aerated for a few hours. It was stored at 0°, and, although generally used fresh, did not lose its activity after several months. The growth from one Roux bottle or one Petri dish generally corresponded to about 10 cc. of the suspension.

The following stock solutions were used: *M*/2 sodium fumarate, *M*/2 sodium *l*-aspartate, *M*/2 sodium *l*-malate, and *M* ammonium chloride. The organic acids were weighed out and neutralised with sodium hydroxide, and all solutions were brought to p_H 7·4. The buffer used was Clark and Lubs's phosphate buffer, p_H 7·4, containing *M*/20 phosphate. Ammonia was estimated on 0·5 cc. samples by the method of Woolf [1928]. Malic acid was estimated polarimetrically as the molybdate compound, by the method of Auerbach and Kruger [1923]. A 5 cc. sample was added to 10 cc. of 14·2 % ammonium molybdate, then 1 cc. of glacial acetic acid was added, and the mixture was

allowed to stand a few hours in the dark. It was then filtered through kieselguhr, exactly 5 cc. of water being used for wetting the filter-paper and washing, so that the total volume of fluid used was 21 cc. The presence of the molybdate helped to precipitate the bacteria, so that a crystal-clear filtrate was obtained. Another 5 cc. sample was added to 11 cc. of 6 % trichloroacetic acid, which was filtered in the same way with the addition of 5 cc. of water. The trichloroacetic acid acted as a protein precipitant and produced about the same degree of acidity as that due to the acetic acid in the molybdate mixture. The two solutions were examined polarimetrically in a 2 dm. tube, and the difference in rotation was proportional to the *l*-malic acid present. It was found that 10 mg. of malic acid, under these conditions, gave a rotation difference, with light from the mercury green line, of + 0·84°. This agrees with the value given by Needham [1927]. All the reaction mixtures were incubated at 37°, the mixtures without inhibitor being contained in filter-flasks evacuated at the water-pump, while those with inhibitor were placed in stoppered flasks, it having been previously ascertained that there was no difference in the course of the reaction anaerobically and aerobically.

Results.

The result of a typical experiment demonstrating the fumarase activity of the organism is shown in Fig. 1. The following reaction mixtures were made up, each containing in addition 50 cc. buffer solution and 2 cc. of *B. coli* suspension.

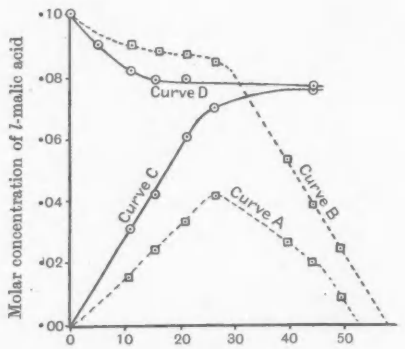

Fig. 1.

Mixture	*M*/2 fumaric cc.	*M*/2 *l*-malic cc.	Propyl alcohol cc.	Water cc.
A	20	—	—	28
B	—	20	—	28
C	20	—	2	26
D	—	20	2	26

Initial malic acid estimations were made, the mixtures were incubated, *A* and *B* being anaerobic, and at suitable intervals further samples were taken for estimation.

Curves *C* and *D* show that in presence of 2 % propyl alcohol an equilibrium is reached when about 76 % of the fumaric acid is converted into malic acid, or 24 % of the malic to fumaric. This equilibrium value agrees with that found by many workers for muscle. The initial portion of curve *C* is linear, as found by Clutterbuck [1927, 1928] for the fumarase of muscle and liver, which suggests that the enzyme is saturated with its substrate for nearly the whole course of the reaction. Curves *A* and *B* show that in the absence of an inhibitor the fumaric-malic equilibrium is masked by some other irreversible reaction which results in the destruction of the malic acid, and follows a linear course. The final portion of curve *A* is invariably parallel to the linear part of curve *B*, but sometimes the curves cross before this part is reached. The same phenomenon is observed in the absence of inhibitors for the aspartic-fumaric

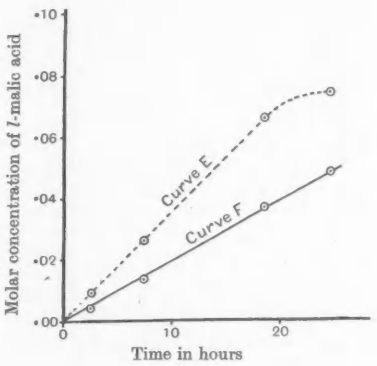

Fig. 2.

equilibrium [cf. Quastel and Woolf, 1926, figs. 1 and 2; Cook and Woolf, 1928, fig. 1], and, as in these cases, the chemical change involved is no doubt a reduction to succinic acid of at any rate part of the malic acid. The matter is under investigation. The figure also shows that the presence of propyl alcohol increases the velocity of the fumarase action. This activation is not always observed.

It was shown by Clutterbuck [1928] that phosphates had an activating action on animal fumarase. This has been confirmed for the enzyme of *B. coli*, as shown by Fig. 2. Curve *E* was obtained with a mixture similar in composition to mixture *C* of Fig. 1, and for curve *F* the buffer was replaced by water. The figure shows that the presence of *M*/40 phosphate at p_H 7·4 almost doubles the reaction velocity. All the other reaction mixtures used in this work contained half their volume of buffer solution.

It is now possible to explain the results of Quastel and Whetham [1924]. Their reaction mixture was similar to that used for curve *A*, save that they used a much higher concentration of *B. coli*. When they examined their solution, at the end of 24 hours' incubation *in vacuo*, it had probably reached the final part of curve *A*, and most of the malic acid previously formed had disappeared, thus leading them to believe that there was very little fumarase in the organism.

Having established the presence in *B. coli* of considerable quantities of fumarase, it became necessary to re-examine the supposed aspartic-fumaric equilibrium. A typical result is shown in Figs. 3 and 4. The reaction mixtures were:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Propyl alcohol cc.	Water cc.
G	20	—	—	2	26
H	—	20	10	2	16
K	—	20	—	2	26

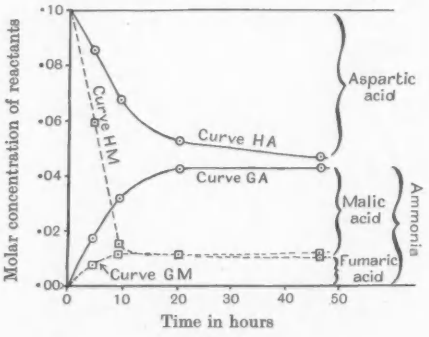

Fig. 3.

Ammonia and malic acid estimations were made on *G* and *H* and malic acid estimations on *K*; the solutions were then incubated and the estimations were repeated at suitable intervals. The results with *G* and *H* are shown in Fig. 3. Curves *GA* and *GM* represent the changes that take place when *M*/10 aspartic acid is incubated with the organism. The distance between *GA* and the base-line represents the molar concentration of free ammonia, found by estimation. This will of course be equal to the sum of the concentrations of fumaric and malic acids. The distance between curves *GA* and *GM* represents the concentration of malic acid, as found by estimation. Hence, by difference, the distance between *GM* and the base-line gives the amount of fumaric acid present, while the aspartic acid concentration is equal to the distance between *GA* and the horizontal line at the top of the figure. Similarly, for the other pair of curves, *HA* denoting free ammonia in *H* and the distance between *HA* and *HM* the malic acid. It will be seen that the ammonia curves *GA* and *HA* are similar

to those given by Quastel and Woolf [1926], but that, at equilibrium, the fumaric acid concentration is only about a quarter of the ammonia concentration, the remaining three-quarters having changed to malic acid, in accordance with the requirements of the fumaric-malic equilibrium.

Fig. 4 is strong evidence that the addition of water and of ammonia to fumaric acid is the work of separate enzymes. It is conceivable that the two reactions could be effected by a single enzyme, which activates fumaric acid, possibly on the lines suggested by Quastel [1926], the activated fumaric acid then "accepting" either water or ammonia, in the form of their ions, which do not need "activation" (Quastel). If this were the case, and one started with a system containing the enzyme and fumaric acid only, then the rate at which malic acid was formed would be governed by the speed with which the

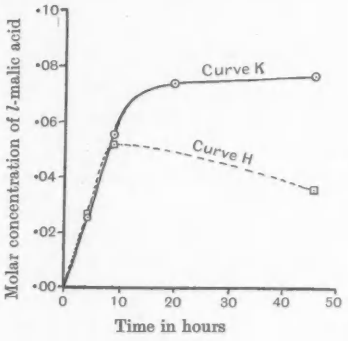

Fig. 4.

enzyme could activate fumaric acid. Now, if ammonia were added to the system, some of the fumaric acid which would have yielded malic acid would be diverted to form aspartic acid; there would be competition between water and ammonia for the activated fumaric acid. On the other hand, if two distinct enzymes were involved, then, so long as the fumaric acid was present in sufficient concentration to saturate the fumarase, the addition of ammonia would not affect the rate of formation of malic acid. Fig. 4 shows that this is in fact the case. Curve *H* shows the rate of malic acid formation from *M*/10 fumaric acid in presence of *M*/10 ammonia, *i.e.* the ordinate at any time equals the distance between curves *HA* and *HM* in Fig. 3. Curve *K* shows the production of malic acid in mixture *K*, which contains no ammonia, and it is clear that the initial rates of malic acid production in the two mixtures are identical.

When malic acid and ammonia are incubated with *B. coli* the final state is the same as that shown in Fig. 3, but the ammonia uptake is slower than with fumaric acid. It is, of course, possible that malic acid goes directly to aspartic acid without passing through the stage of fumaric acid. That this is unlikely is shown by Fig. 5. Mixture *L* contains *M*/10 fumaric acid and *M*/10

ammonia, together with a very small quantity of organism, while in mixture *M*, malic acid is substituted for the fumaric acid. The curves show the details of the beginnings of the ammonia uptakes. Fumaric acid immediately begins to react, but with malic acid there is a lag, indicating that fumaric acid must be formed before the ammonia uptake can begin.

The equilibrium constant K_1 for the reaction

$$COOH.CH = CH.COOH + H_2O \rightleftharpoons COOH.CH_2CH.OH.COOH$$

is given by the equation

$$K_1 = \frac{[\text{Fumaric acid}]\,[\text{Water}]}{[\text{Malic acid}]}.$$

The concentration of water can be taken as constant. Hence the ratio of fumaric acid to malic acid at equilibrium will be constant, whatever their initial concentrations may be. Now in the aspartic-fumaric equilibrium the constant K_2 is given by

$$K_2 = \frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}.$$

The value found for this constant by Quastel and Woolf [1926] was 0·04, but what they supposed was the fumaric acid concentration was really the sum of the fumaric and malic acid concentrations. The true concentration of fumaric acid is only a quarter of the value they used. It is this constancy of the

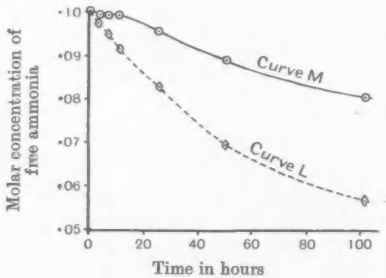

Fig. 5.

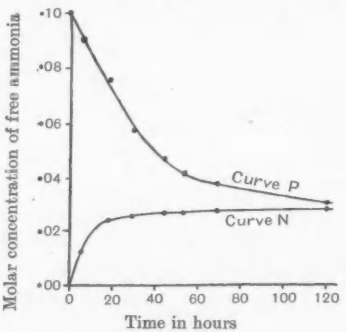

Fig. 6.

fumaric-malic ratio that enabled them to find the same value of K for the complex equilibrium when they varied the concentrations of their reactants. Substituting the true value of the fumaric acid concentration in the equation for K_2, one finds that K_2 is about 0·01. From this value it can easily be calculated that, if the fumaric-malic reaction could be entirely eliminated, the equilibrium mixture for the aspartic-fumaric equilibrium, when the reactants were present in the usual initial concentrations of *M*/10, would contain only about 28 % of free ammonia instead of the 47 % found for the mixed equilibrium.

Fig. 6 shows the result of an experiment in which these conditions are realised. The effect of cyclohexanol on the fumarase of *B. coli* is peculiar. If

the organism is incubated with 2 % cyclohexanol for an hour or two, the fumarase is markedly activated, but if the incubation is continued for 15 hours, the fumarase activity is sometimes found to have entirely disappeared. This is not always the case, and it is probable that the time required for the destruction of the enzyme varies for different strains and preparations of the organism. When the fumarase is destroyed in this way it is found that malic acid will show no ammonia uptake, but ammonia uptake by fumaric acid still occurs. In the experiment to be described, 20 cc. of stock *B. coli* suspension were added to 78 cc. of saline and 2 cc. of cyclohexanol, and the mixture was incubated for 15 hours, when the fumarase was found to be completely destroyed. The following reaction mixtures were then made up:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Buffer cc.	Cyclo-hexanol cc.	Treated *B. coli* cc.	Water cc.
N	20	—	—	50	1·8	10	18·2
P	—	20	10	50	1·8	10	8·2

The mixtures were incubated, and ammonia and malic acid estimations made at intervals. The malic acid estimations were negative, and the ammonia results are shown in Fig. 6. It will be seen that the final value obtained is the true one for the aspartic-fumaric equilibrium.

This differential destruction of the mechanisms responsible for the two reactions is a further proof that two distinct enzymes are involved, and the absence of ammonia uptake by malic acid after the destruction of fumarase confirms the conclusion that malic acid itself is not converted into aspartic acid. It is convenient to have a name for the enzyme responsible for the aspartic acid reaction and it is proposed that it be provisionally termed *aspartase*.

It was found by Alwall [1928] that the fumarase in a succinoxidase preparation from muscle is destroyed by incubation at 50° for half an hour. Boiled *B. coli* shows no fumarase or aspartase activity, and preliminary experiments indicate that the inactivation temperature for each enzyme is in the neighbourhood of 50°.

Discussion.

B. coli communis is able to bring about several chemical reactions involving fumaric acid. One is the irreversible reaction shown in Fig. 1, which is inhibited by propyl alcohol. Possibly another is the oxidation of fumaric acid, though it seems probable that this may only take place *via* malic acid. Finally there are three reversible reactions: the fumaric-succinic-methylene blue equilibrium [Quastel and Whetham, 1924], and the equilibria governed by fumarase and aspartase. Each of these reactions seems to be catalysed by a distinct enzyme, which can effect one reaction only. Working with mammalian tissues, Alwall [1928] destroyed fumarase and left succinoxidase, and Clutterbuck [1928] destroyed succinoxidase and left fumarase. It has been shown in this paper that the fumarase of bacteria can be destroyed without elimination of aspartase. Quastel and Wooldridge [1927, 1] showed that the succinoxidase of

B. coli is destroyed by incubation with cyclohexanol for 5 minutes, whereas fumarase has been shown above to be far more resistant to this substance. They also showed that succinoxidase is little affected by one hour at 57°, a treatment that would destroy both fumarase and aspartase. Quastel and Wooldridge [1928] report that, after treatment with toluene, the affinity of the succinoxidase of *B. coli* for malonic acid is vastly increased, so that small concentrations have a large effect in retarding the reaction velocity. Experiments made on fumarase and aspartase show that they do not possess this property. Finally, repeated attempts have been made to demonstrate the presence of aspartase in muscle, with uniform lack of success, although the various preparations used showed marked fumarase activity.

It seems clear, therefore, that three reactions as similar as the addition across the double bond of fumaric acid of 2H, H and OH, and H and NH_2, require separate enzymes for their catalysis. Now according to the "activation" theory of Quastel [1926], as modified and extended by Quastel and Wooldridge [1927, 2] into the "active centre" theory, one would expect the essential happening in all these reactions to be the same, the activation of fumaric acid. The fumaric acid would be adsorbed or combined at an active centre, at which it would come under the influence of an intense electric field, becoming activated, so that its state could be diagrammatically represented as $COOH.CH_2.\check{C}.COOH$. The activated molecule would then react with ions from the solution, which do not need activation, and which would combine at the temporarily unsaturated or active carbon atom represented by the sign $\check{C}$. If this were the true mechanism, one would expect all three reactions to be effected by a single enzyme; or, if it were objected that the three reactions required fumaric acid activated to different extents, then it would be anticipated that the enzyme that could effect the most difficult of the three reactions —the one requiring the highest energy of activation or the most intense electric field—should also be able to bring about the other two reactions, while the enzyme catalysing the more difficult of these two reactions should also bring about the easier, and only the enzyme with the weakest field should be truly specific for one reaction only. But experiment shows that the contrary is the case; each enzyme is specific for one type of reaction. The differences in the enzymes seem not to be merely quantitative, as one would expect if the views of Quastel and Wooldridge were accepted as adequate, but there appear to be qualitative differences between them.

At the present stage it is only possible to give the merest indications of what the nature of these qualitative differences may prove to be. It will be noticed in curve *HA*, Fig. 3, that the initial portion is linear for a large portion of the reaction. This suggests that the enzyme is working at full saturation with its substrate. Now the reaction taking place involves two substances, fumaric acid and ammonia. If it were supposed that the ammonia were acting in solution, combining with activated molecules of fumaric acid at the enzyme when it came into contact with them by collision, then, by the law of mass

action, it would be expected that, as the ammonia concentration fell, the reaction velocity would fall proportionately, even although the enzyme was saturated the whole time with fumaric acid. One would only expect to get a linear reaction if the enzyme were saturated with both the reactants—fumaric acid and ammonia. It follows that one must suppose that ammonia is combined at the enzyme as well as fumaric acid. Similar considerations apply to the reduction of methylene blue by succinic acid in presence of bacteria. Quastel [1926] gives a figure (Fig. 1) showing that the rate of decoloration is linear during the greater part of the reaction. This implies that the enzyme is saturated with methylene blue, and therefore that there is combination between enzyme and dye. Further evidence leading to the same conclusion is furnished by the work of Dixon [1926] on xanthine-oxidase and Quastel and Wooldridge [1927, 1] on several of the dehydrogenating enzymes of *B. coli*. These workers used various dyes of different reduction potentials, and found that the rate of reaction was not dependent merely upon the ease of reduction of the indicator. Dixon points out that the presence of sulphonic groups in the dye tends to slow its reduction rate. These facts suggest that the chemical nature of the dye affects its affinity for the enzyme, and hence its rate of reaction. Quastel and Wooldridge themselves state that "the velocity of reduction is governed by the concentration of indicator as well as by the concentration of activated donator at the surface," but they do not seem to mean that the combination between enzyme and indicator is of the Michaelis type, nor do they seem to the writer to bear this condition sufficiently in mind in the development of their theory.

These considerations, and similar ones applied to the facts known about some other enzymes, point to the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succin-oxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase. Whether there is also activation by electric fields as Quastel postulates, or whether the mechanism by which reaction is effected is of a different nature, seems to the writer still a very open question. This hypothesis has at any rate the merit that it is capable of being tested on strictly quantitative lines, and it is hoped to carry out further studies on fumarase and aspartase with this object in view.

Summary.

1. The presence is demonstrated in *B. coli communis* of considerable quantities of fumarase, the enzyme governing the equilibrium between fumaric acid and *l*-malic acid. In the absence of inhibitors, such as 2 % propyl alcohol, the action of this enzyme is masked by an irreversible process resulting in the anaerobic destruction of malic acid. In presence of propyl alcohol the same equilibrium is attained as that given by animal fumarases.

2. The equilibrium previously reported between aspartic acid, fumaric acid and ammonia is really a complex malic-fumaric-aspartic equilibrium. The fumarase of *B. coli* can be destroyed by treatment of the organism with cyclohexanol, and then the true aspartic-fumaric equilibrium is obtained.

3. The three reversible changes involving fumaric acid—those to succinic, malic and aspartic acids—are catalysed by distinct enzymes. It is proposed to call the enzyme responsible for the third of these reactions *aspartase*.

4. The bearing of these results on enzyme theory is discussed, and a limited hypothesis on enzyme action is put forward.

It is a pleasure to express my gratitude to Sir F. G. Hopkins and Mr J. B. S. Haldane for their continued encouragement during the course of this work.

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LV. THE DISTRIBUTION OF VITAMIN B_2 IN CERTAIN FOODS.

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By vitamin B_2 is here meant the factor in the vitamin B complex, other than the antineuritic, which promotes growth and cures and prevents dermatitis in rats. Its possible identity with the "P. P." factor, preventive and curative of human pellagra, and probably of "black-tongue" in dogs, is discussed later in this paper.

Since the discovery that McCollum and Davis's [1915, 1, 2] water-soluble B vitamin can be subdivided into at least two factors, Eijkman's [1897] antineuritic and another [Smith and Hendrick, 1926; Goldberger *et al.*, 1926; Chick and Roscoe, 1927], no attempt has been made to work out systematically the distribution of the more heat-stable factor, vitamin B_2. The distribution of the other constituent, the antineuritic vitamin B_1, as investigated by the power of a food to protect a bird on polished rice from polyneuritis or to cure the condition when it is developed, is presumably not affected by the discovery of another factor in the vitamin B complex. As early as 1912 Cooper [1912] showed that pigeons require to maintain their weight on a diet of polished rice a factor different in its distribution from that which protects against and cures polyneuritis, and it is possible that this factor was what is here designated vitamin B_2. But, since autoclaved yeast, one of the richest sources of vitamin B_2, has no effect in curing polyneuritis, it would follow that the presence or absence of vitamin B_2 in the food does not affect its strictly antineuritic potency. The distribution of the thermolabile antineuritic factor B_1, in so far as it has been investigated by experiments with birds fed on polished rice, has therefore not been upset by later discoveries.

Where the "water-soluble vitamin B" value of a substance has been investigated with the growth of rats as a criterion, it is not possible, since both factors are equally necessary for growth, to form any idea of the relative amounts of vitamins B_1 and B_2 present. Chick and Roscoe's [1928] work showed that it is difficult to clear caseinogen completely of vitamin B_2, and it may reasonably be assumed that most "vitamin B-free" diets have hitherto not been free of this factor. Moreover, it is not until recently that techniques have been evolved for supplying a rat with vitamin B_1 without vitamin B_2.

Distribution of vitamin B_2 as indicated by previous observations on the growth of rats.

In the course of previous work demonstrating the dual nature of vitamin B, some indications of the distribution of vitamin B_2 have been given. This factor is abundant in yeast, fresh or autoclaved [Smith and Hendrick, 1926; Chick and Roscoe, 1927; Sherman and Axtmayer, 1927]; 0·2 g. daily of fresh yeast and 0·4 g. of autoclaved maintained growth in rats receiving daily an antineuritic yeast extract free from vitamin B_2 [Chick and Roscoe, 1927]. Sherman and Axtmayer [1927] found when observing growth in rats on a vitamin B-free diet that whole wheat supplemented autoclaved yeast, and dried skim milk supplemented whole wheat, and drew the conclusion that whole wheat is richer in vitamin B_1 (F) than in vitamin B_2 (G), while with milk the reverse is the case. Hunt and Kraus [1928] found that 5–10 cc. of fresh whole milk daily supplemented, as source of "vitamin B," a yeast-fraction which could also be supplemented by autoclaved yeast. A number of observations on pigeons have also indicated that milk is a poor source of the antineuritic vitamin [Cooper, 1914; Gibson and Conception, 1916; Johnson and Hooper, 1921].

Chick and Roscoe [1927] observed that it required a daily dose of 1 g. of wheat embryo to produce normal growth in conjunction with an antineuritic concentrate prepared according to Peters's method [Peters, 1924; Kinnersley and Peters, 1925], whereas 0·2 g. daily of the embryo could produce good growth when supplemented by autoclaved yeast.

McCollum, Simmonds and Pitz [1917] state, without giving experimental results, that the antineuritic value of the oat kernel is high; by analogy with other cereals one would expect this to be the case. Smith and Hendrick [1926], however, found that a diet containing 40 % of whole oats failed to produce normal growth in rats unless supplemented by 5 % fresh or autoclaved yeast, suggesting that oatmeal is richer in vitamin B_1 than in vitamin B_2.

Salmon [1927] obtained results suggesting that the leaves of the velvet bean were richer in vitamin B_2 than the beans themselves, while the distribution of vitamin B_1 was the opposite.

Goldberger *et al.* [1926] reported that rats fed on a diet containing 20 % of dried fresh beef as the sole source of vitamin B rapidly declined and died, with or without polyneuritis. 5 % of an alcoholic maize extract, which, supplemented by autoclaved yeast, could produce good growth, but of which alone as much as 40 % produced no growth, was added. With this addition the rats grew in a normal manner, suggesting that dried beef is a stronger source of vitamin B_2 than of vitamin B_1.

The results of these investigations can be roughly summarised as follows. Unheated yeast is rich in both vitamins B_1 and B_2; autoclaved yeast only in vitamin B_2. The cereals contain more vitamin B_1 than vitamin B_2, milk and meat the reverse. In the velvet bean the leaves are richer in vitamin B_2, the seeds in vitamin B_1. It is impossible to make any quantitative estimates of vitamin B_2 from these observations.

Experimental.

In the present research a thorough investigation of the wheat and maize kernels for their vitamin B_2 content was made; the point of interest being to determine, since human pellagra is a maize, rather than a wheat, eater's disease, any significant difference in vitamin B_2 content of the two cereals. We have also investigated certain other common foodstuffs in a less exhaustive fashion.

Method used for assaying vitamin B_2.

The technique elaborated by Chick and Roscoe [1928] was used. In this method B_1 is supplied to the rats as Peters's antineuritic concentrate, while special efforts are taken to free the caseinogen in the basal diet from vitamin B_2 by thorough washing with acidulated water and extraction with dilute acid alcohol. It is difficult to check all growth in rats and induce pellagra-like symptoms in them unless this method of purification is followed.

Our basal diet P_2L was as follows:

Specially purified caseinogen	20 parts
Rice starch	60 ,,
Cotton-seed oil	15 ,,
Salt mixture[1]	5 ,,
(Water	100 ,,)

The diet was cooked in a steamer for 3 hours at 100° to prevent refection [Fridericia *et al.* 1927; Roscoe, 1927].

Diet P_2L, with the daily addition of 0·05–0·1 g. of cod-liver oil to provide vitamins A and D, and 0·1 cc. (equivalent to 0·6 g. yeast) daily of Peters's antineuritic concentrate [Peters, 1924; Kinnersley and Peters, 1925], to provide vitamin B_1, was assumed to supply all the factors necessary for rat growth except vitamin B_2 (or the part of the vitamin B complex other than the antineuritic).

In the investigation of the cereals the materials under examination were mixed into the basal diet in such proportions that the standard ratio between protein, carbohydrate, and fat (20 : 60 : 15) was not altered, using for the calculation the commonly accepted analyses of the cereals. For example, white flour roughly contains: protein 10 %, carbohydrate 90 %; in constructing a diet containing 65 % of white flour, the following proportions were accordingly employed:

Purified caseinogen	13·5 parts	
Rice starch ...	1·5 ,,	
Cotton-seed oil ...	15·0 ,,	
Salt mixture ...	5·0 ,,	
White flour ...	65·0 ,,	(6·5 parts protein; 58·5 parts carbohydrate)
(Water ...	100·0 ,,)	

making the percentages of protein, etc., similar to those in the basal diet P_2L. The composition of other cereal diets was similarly calculated. The diets were cooked for 3 hours at 100°. We did not in any case allow the cereal protein to replace more than one-half the caseinogen of the original basal diet; in most cases it replaced about one-third.

In the investigation of other foods, of which the necessary quantities were small, daily additions were made to the basal diet P_2L.

Young rats, just weaned, from 30 to 45 g. in weight, were fed for a week on diet P_2L, with the addition of three drops (0·05 g.) of cod-liver oil daily to provide vitamins A and D, Peters's antineuritic concentrate being omitted for economy's sake. At the end of the week, during which growth always ceased, the rats were put in separate cages and the diet containing the substance under examination was fed to them, 0·1 cc. (=0·6 g. dry yeast) daily of Peters's antineuritic concentrate (vitamin B_1) being now added. One rat from each litter, usually the best grown, was kept on diet P_2L with additions of cod-liver oil and antineuritic concentrate only, to act as a "negative control."

[1] Salt mixture No. 185 [McCollum, Simmonds and Pitz, 1917].

Young rats, when first caged singly and put on a strange diet, take a few days to get used to new conditions. Very little growth may take place in the first week, even on a good diet. We therefore disregarded the first week's growth, and took for our standard of comparison the average weekly growth during the succeeding 4 weeks, the vitamin B_2 value of a foodstuff being estimated by the minimum daily ration needed to maintain normal increase in body-weight (11–14 g. weekly) during those 4 weeks. In a few cases when, after a test period of 2 to 3 weeks, it became obvious that the diet contained insufficient vitamin B_2 for growth, we increased the amount of test substance in the ration and began again, using the succeeding 4 weeks for our standard of comparison.

We found sometimes a different response to the same diet by rats from different litters. This difference was not large enough to produce markedly incompatible results, but made it necessary to distribute each test material through several litters, so that in no case should conclusions be drawn from results obtained on rats of one litter only. Males and females were used in approximately equal numbers in testing each foodstuff.

Where it seemed of interest to compare as accurately as possible the vitamin B_2 content of certain foods, such as "household" and "top patent" flour and whole wheat and whole maize, rats from the same litter were used, as these give a more uniform response.

The power of certain foodstuffs to cure dermatitis which had developed in rats on the basal diet was also tested (Table V).

1. *The vitamin B_2 content of various samples of wheat, maize and peas, tested as different proportions of the diet.*

Two kinds of whole wheat were tested, (A) a mixture consisting mainly of Manitoba wheat, (B) a sample of English wheat. Of sample (A) five different parts of the berry were tested, viz. (1) the germ, which in this sample appeared to be quite free of bran, (2) the bran, (3) "top patent" flour, a sample specially collected and consisting of almost pure endosperm, (4) "household" flour, a coarser article than the "top patent" and one that is in commercial use, and (5) "tails" or fine pollard, a mixture of all parts of the berry carrying a high percentage of bran. The last was tested with the idea that it might represent a more or less pure specimen of the aleurone layer which lies between the bran and the endosperm, but on further investigation the sample was found to be too mixed for such conclusions to be drawn.

Two samples of whole maize were tested, (A) white African maize, (B) yellow South American maize. Maize germ meal and maize "grits" from sample (B) were also investigated. The maize germ meal contained a fair amount of endosperm, the maize "grits" was, as far as was ascertainable, fairly pure maize endosperm. "Polenta," another form of maize endosperm, was also tested.

In view of the extreme importance of the comparison of whole wheat and whole maize, an additional experiment was set up in which the two samples of whole wheat and the two samples of whole maize were tested on rats of similar weights from the same litter. The result of this experiment is shown in Fig. 1.

A sample of dried peas was tested.

The "polenta" and the peas were bought across the counter, the other samples being obtained direct from the mills.

The results of these experiments are shown in Table I, and in Table II are given the minimal amounts which must be present in the diet if normal growth is to be maintained.

Table I. *Average weekly growth increments of rats on:* A, *a mixed diet;* B, *purified diets, deficient in vitamin* B_2, *and with this vitamin supplied by autoclaved yeast;* C, *synthetic diets in which vitamin* B_2 *is supplied by various percentages of cereal products and peas.*

(Vitamin B_1 supplied in B and C by daily doses of Peters's antineuritic concentrate from yeast.)

	Material tested	No. of rats observed	Average weekly increase in body-weight for chosen 4 weeks (g.)
A	Mixed diet. Dried or fresh winter milk, white or brown bread, cabbages or carrots. With Quaker oats, wheat bran, sheep's lungs and cods' heads once or twice a week	16 stock rats from 5th–9th week of life	14 ♂ 16; ♀ 12.
B	Negative controls. Diet P_2L	15	2
	Positive controls. Diet P_2L + autoclaved yeast (0·4 g. daily)	5	12·5
C	Wheat. Whole ground wheat. Sample A, Manitoba, 30 %	2	5·5
	50 %	10	16
	Sample B, English 50 %	4	12
	Wheat germ. Sample A, 7·5 %	4	2·5
	15 %	6	9
	30 %	6	16
	Wheat bran. Sample A, 15 %	4	8
	30 %	4	13·5
	Wheat "tails" (fine pollard). Sample A, 15 %	4	7·5
	30 %	4	15
	Flour. Top patent. Sample A, 65 %	7	5
	Household. Sample A, 65 %	4	5
	Maize. Whole ground maize. Sample A, white, African, 30 %	4	8·5
	50 %	6	11
	Sample B, yellow, S. American, 50 %	8	8
	Maize germ meal. Sample B, 15 %	4	2
	30 %	3	7
	60 %	6	13
	Endosperm. Maize "grits." Sample B, 65 %	6	5
	"Polenta," Italian, 65 %	4	4
	Peas. Dried ground "Clipper" peas, 30 %	2	9·5
	45 %	5	16

Whole wheat cannot be regarded as a rich source of vitamin B_2, since 30 % in the diet of sample A, a mixed sample consisting chiefly of Manitoba wheat, proved insufficient for growth; with 50 % of this wheat in the diet excellent growth was obtained. The sample of English wheat (B) was not as good a source of the vitamin; with 50 % in the diet growth was only just normal.

Wheat endosperm, either as "top patent" flour or as the less pure "household" flour, is a poor source of vitamin B_2, 65 % of either produced very little growth. Wheat embryo and bran are about equal to each other in their vitamin B_2 potency, and to wheat "tails"; in each case 15–30 % provides sufficient vitamin for normal growth. Tibbles [1912] gives the composition of the wheat berry as follows: bran 13·5 %, endosperm 85·0 %, germ 1·5 %, so that, in the wheat kernel as a whole the bran is the most important source of vitamin B_2.

Table II. *Minimum amounts of cereal products, given incorporated in the diet, required to provide enough vitamin B_2 for normal growth in young rats (11–14 g. weekly increase in body-weight).*

Material			Amount required % of diet
Wheat.	B, English.	Whole grain	50
	A, Manitoba.	Whole grain	30–50
		Embryo	15–30
		Bran	15–30
		Pollard	15–30
		Patent flour	>65
		Household flour	>65
Maize.	A, white, African.	Whole grain	50
	B, yellow, S. American.	Whole grain	>50
		Germ meal	30–60
		Endosperm "grits"	>65
	C, endosperm Italian "polenta"		>65
Peas.	Dried "Clippers"		30–45

N.B. Where two figures are given the lower was insufficient.

The mixed wheat sample, Manitoba predominating, was typical of the wheat mixtures used in London flour mills. It must be remembered, however, that since our investigation was made on particular samples, it is impossible to assume that these are representative.

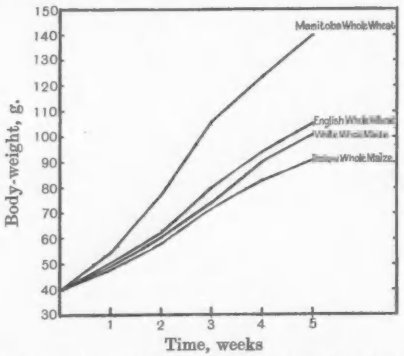

Fig. 1. Comparison of growth-promoting value of two samples of whole maize and two of whole wheat, when given as the only source of vitamin B_2 in an otherwise complete diet. The curves show the average growth of two rats (1 ♂ and 1 ♀) on each diet. 50 % of the cereal product was included in the diet in each case.

Whole yellow maize is definitely inferior in its vitamin B_2 content to either of the samples of whole wheat tested. With 50 % in the diet the rats did not grow normally. The sample of white maize tested had a higher vitamin B_2 value than the yellow sample. The same amount, 50 %, in the diet was needed to produce normal growth as was needed of English wheat; it was, therefore, much inferior to the mixed Manitoba wheat (see Fig. 1).

Maize endosperm, as "grits" or as "polenta," is very low in the vitamin, as is wheat endosperm; with 65 % in the diet the rats scarcely grew at all. The maize germ meal used was a less cleanly milled product than the wheat germ. In the maize kernel the germ is attached to the endosperm by a large

scutellum and can only be prepared in the pure state by careful hand dissection. Maize germ meal, therefore, carries of necessity a large proportion of both endosperm and scutellum. Even taking this into account, the maize germ may be considered a poorer source of vitamin B_2 than wheat germ, as 60 % was needed to produce a normal rate of growth as against 30 % of wheat germ.

Vitamin B_2 and the antineuritic vitamin are differently distributed in the wheat and maize kernels. Vitamin B_2 is not entirely absent from the endosperm as is vitamin B_1. The rats on the 65 % white flour, and 65 % maize "grits," diets grew slightly better than those on the basal diet and did not develop pellagra-like symptoms except in one case, when a rat on 65 % "top patent" flour developed mild dermatitis. As regards the antineuritic vitamin B_1 "the germ, weight for weight, was found to be five times as potent as the bran in the cure of polyneuritis" [Medical Research Council, 1924]. In contrast with this, the vitamin B_2 content of the bran and the germ are about equal.

The vitamin B_2 value of dried peas cannot be considered high, 45 % in the diet being necessary before normal growth was obtained.

2. *The vitamin B_2 content of various foodstuffs, tested as additions to the basal diet.*

Yeast, dried and autoclaved for 5 hours at 120°, fresh whole winter milk from the ordinary London supply, cooked dried egg yolk, dried beef steak and dried ox liver were tested for their vitamin B_2 content by feeding small doses daily to rats which received the basal diet with the addition of Peters's yeast concentrate as source of vitamin B_1.

Table III. *Average weekly growth increments of rats on a purified caseinogen diet deficient in vitamin B_2 (P_2L), supplemented with daily doses of various foods.*

(Vitamin B_1 supplied by daily doses of Peters's antineuritic concentrate from yeast.)

Material tested	Amount given daily: Air dry g.	Amount given daily: Natural state	No. of rats observed	Average weekly increase in body-weight for chosen 4 weeks (g.)
Liver (dried ox liver)	0·06	0·2 g.	2	4
	0·12	0·4	3	12
	0·25	0·8	1	18·5
Yeast (dry)	0·1	0·5 g.	2	8
	0·2	1·0	3	16
Yeast (autoclaved)	0·4	2·0 g.	5	12·5
Milk (fresh whole, London winter supply)	0·36	3 cc.	3	9
	0·72 solids	6	3	12
	0·96	8	2	14
Egg yolk (cooked, dried)	0·5	1 g.	2	7
	1·0	2	4	11
Meat (dried steak)	0·25	13	2	3·5
	0·5	2	3	9
	0·75	3	3	13
	1·0	4	2	15

The results of this experiment are given in Table III, and in Table IV are shown the minimum amounts which must be given if normal growth is to be maintained.

Table IV. *Minimum amounts of substances containing vitamin B_2 given as daily additions to a basal diet (P_2L), required for the normal growth of young rats (11–14 g. weekly increase in body-weight).*

Material	Daily dry weight g.	Daily amount as natural foodstuff
Liver (dried ox liver)	0·06–0·12	0·2–0·4 g.
Yeast (dried)	0·1 –0·2	0·5–1·0 g.
Yeast (autoclaved)	0·4	2·0 g.
Milk (fresh whole, London winter supply)	0·36–0·72	3–6 cc.
Egg yolk (cooked, dried)	0·5 –1·0	1–2 g.
Meat (dried steak)	0·5 –0·75	2–3 g.

N.B. Where two figures are given the lower was insufficient.

All these natural foodstuffs enabled rats to increase normally in weight when given in doses of 1 g. and under.

Since we kept a daily record of the food consumption of each animal, it is possible to draw rough comparisons between the foods which were incorporated as different percentages of the diet and those fed as additions to it. Rats growing at a normal rate on diet P_2L (with additions) consumed from 6 to 7 g. (dry weight) daily. A dose of 1·0 g. dry weight would therefore represent 14–12·5 % of the dry weight of the total food consumed, one of 0·5 g. 7–6 %. Normal growth was obtained on the cereal diets only when 30 % or more of the cereal product was included in the total dry weight consumed, or when 2 g. and upwards of the cereal product was consumed daily.

The substances tested in Table III are therefore to be reckoned better sources of vitamin B_2 than the cereal products and peas, if the comparison is made on the dry weights of the substances concerned.

The following rough comparison of the foodstuffs tested may therefore be made, the substances being placed in descending order as regards their B_2 content, this being reckoned on the dry weight.

1. Dried ox liver
2. Dried yeast
3. Autoclaved yeast
4. Meat (dried steak)
5. Milk solids
6. Dried egg yolk
7. Wheat germ
8. ,, bran
9. ,, pollard
10. Dried peas
11. Whole wheat (Manitoba)
12. ,, ,, (English)
13. Whole maize (white)
14. ,, ,, (yellow)
15. Maize germ meal
16. Wheat endosperm
17. Maize endosperm

3. *The curative effect of various foodstuffs on the dermatitis and other symptoms developing in rats on a vitamin B_2-deficient diet.*

Of 15 rats from 15 different litters kept as negative controls on diet P_2L with the addition only of cod-liver oil and antineuritic concentrate, none showed significant growth, and 9 developed symmetrical dermatitis. Of these 8 displayed lesions of the fore-paws, the hind feet also being affected in 6 cases and the groins and axillae in 3; one rat showed dermatitis of the groins and axillae alone. The axillary lesions tended to spread down the inner side of the forelimbs and across the chest and those in the groins down the inner side of the thighs. The dermatitis of the extremities was usually accompanied by oedema, which in 2 rats was so severe as to cause gangrene by constriction, with the loss of six toes in one case. The general condition of the rats showing skin symptoms was usually miserable.

The average time taken for symptoms to appear was 10 weeks, the shortest period being 5 weeks, and the longest 22.

The remaining 6 rats deprived of vitamin B_2, which showed no localised dermatitis, developed nevertheless a condition of general wretchedness after a period varying from 5 to 8 weeks. They showed a mixture of such symptoms as ophthalmia, inflamed nostrils, loss of hair on head and elsewhere, and haematuria. These symptoms were equally frequent in the rats showing definite dermatitis.

The symptomatology was as described in a previous paper from this laboratory [Chick and Roscoe, 1928], except that skin symptoms developed more constantly. A curious feature was the tendency of certain symptoms to predominate at certain times. At one period in the autumn the sick rats tended to develop severe lesions of the extremities and of the eyes, while later the groins and axillae were most often affected, the eyes being almost normal.

The power of certain foodstuffs to cure these symptoms of dermatitis was tested on eight of these negative control rats and on four other similar rats, negative controls from other experiments.

In addition to these young rats, two adult rats, aged about 1 year, were put on the vitamin B_2-free diet. One of these (801 ♀) dropped in weight from 350 to 170 g. in 11 weeks, and died without showing skin symptoms, though developing some ophthalmia. The other (802 ♂) dropped from 200 to 140 g. in 10 weeks, its weight then became stationary and at the end of 12 weeks it began to show dermatitis, which rapidly became very severe. This rat was used for a curative experiment.

The results of the curative experiments are given in Table V.

In these experiments it was found that the growth and healing properties of the substances tested ran parallel. The foodstuffs were mostly the same as those of which the vitamin B_2 content had been tested by growth experiments. The results confirm the idea that in the rat a growth-promoting and a dermatitis-preventing factor are identical. In addition to the substances used in the

Table V. *The curative effect of various substances on the dermatitis and other symptoms developing in rats on a vitamin B_2-deficient diet (P_2L).*

Rat	Condition before test	Substance tested	Length of time substance was fed	Result
771 ♀	Ophthalmia. Wretched condition. Weight stationary	Cheddar cheese 1 g. daily	3 weeks	Very slight improvement; 9 g. increase in wt.
714 ♂	Severe ophthalmia. Wretched condition. Dermatitis of extremities. Growth stationary	Cheddar cheese 2 g. daily	3 weeks	All lesions healed; 28 g. increase in wt.
711 ♀	Poor general condition. Dermatitis of axillae, groins and feet. Cessation of growth	Fresh meat 0·4 g. daily (0·1 g. dry wt.)	2 weeks	Improvement in dermatitis; 5 g. increase in wt.
734 ♀	Severe dermatitis of axillae and groins. Poor general condition. Cessation of growth, loss of weight	Cooked liver 0·4 g. daily (0·12 g. dry wt.)	3 weeks	Complete cure; 37 g. increase in wt.
3061 ♂	Severe dermatitis of fore-paws. Ophthalmia. Nose inflamed. Haematuria. Cessation of growth	Wheat germ 7½ %	1 week	No improvement or resumption of growth
		Wheat germ 15 %	1 week	Ditto
		Wheat bran 15 %	1 week	Ditto
		P_2L + 1·0 cc. yeast fraction ≡ 5 g. dry yeast	2 weeks	Complete cure; 28 g. increase in wt.
3067 ♂	Dermatitis of fore-paws. Inflamed nose. Haematuria. Cessation of growth	Patent flour 65 %	5 days	Died
378 ♂	Severe ophthalmia. Diarrhoea. Cessation of growth	Patent flour 65 %	3 weeks	Temporary improvement, followed by regression; no growth
		P_2L + 0·4 g. autoclaved yeast	3 weeks	Complete cure; 47 g. increase in wt.
728 ♂	Dermatitis of tail and extremities. Commencing gangrene of two toes. Cessation of growth	Maize germ meal 60 %	3 weeks	Toes sloughed off and all dermatitis healed; 30 g. increase in wt.
748 ♂	Severe dermatitis of fore-paws and feet. Commencing gangrene of toes. Dermatitis of tail. Weight stationary	Maize germ meal 60 %	3 weeks	Complete cure of dermatitis; toes sloughed off and stumps practically healed; 31 g. increase in wt.
729 ♂	Severe dermatitis of fore-paws. Weight stationary	Milk 6 cc. daily	3 weeks	All lesions healed; 38 g. increase in wt.
802 ♂	Great wasting. Dermatitis of axillae and groins, spreading down inside of thighs and forelimbs. Large raw area on abdomen	Egg-white 5 g.	3 weeks	Complete cure of all lesions; 31 g. increase in wt.
3075 ♀	Ophthalmia. Slight dermatitis of fore-paws. Cessation of growth	Egg-white 60 % of diet	3 weeks	Complete cure; 42 g. increase in wt.
757 ♀	Ophthalmia, very poor condition. Severe dermatitis of groins and axillae. Haematuria. Loss of weight	Egg-white extract (≡5 g. fresh egg-white)	4 weeks	Complete cure of all symptoms; 26 g. increase in wt.

growth experiments, Cheddar cheese and egg-white were tested. The former was not strongly curative as compared with milk; 2 g. daily of cheese (1·3 g. dry weight) was needed as compared with 6 cc. (0·7 g. dry weight) of milk. Egg-white was more potent; 5 g. (0·6 g. dry weight) produced a rapid amelioration of symptoms.

We have occasionally observed a very slow amelioration of symptoms taking place in rats kept throughout on the basal diet without any vitamin B_2 additions. In the above experiments, however, when improvement followed alterations in the diet it was striking and immediate and left little doubt of its cause.

Discussion of the relation of vitamin B_2 to the vitamin B complex, to the "P. P." (pellagra-preventive) factor, and to the factor preventing black-tongue in dogs.

It is by no means proved that vitamin B_2 is the only factor in the vitamin B complex other than that contained in Peters's antineuritic concentrate and several workers have brought forward evidence pointing to the existence of a third factor.

Hunt [1928] observed that an absorbable fraction (? vitamin B_1) and a filtrate (? vitamin B_2) from yeast, which could respectively supplement autoclaved yeast and whole wheat, could be further supplemented by whole fresh yeast or yeast residue minus the above fractions. We do not think, however, that in these experiments the possibility of a quantitative deficiency of either fraction was sufficiently excluded. The same criticism applies to Reader's observations [1928] that growth failed in rats whose vitamin B was supplied by Peters's antineuritic concentrate and large amounts of autoclaved marmite. Chick and Roscoe [1927] find that Peters's yeast concentrate in an amount equivalent to 0·6 g. yeast daily if supplemented by 0·4 to 0·6 g. autoclaved yeast supports normal growth.

We ourselves are more concerned with the possibility that in our experiments lack of growth and skin symptoms may not be due to the absence of the same factor. We can only say that those foods which promoted fair or good growth in young rats also protected from skin symptoms, and that in the curative experiments growth and healing ran parallel. The fact, however, that vitamin B_2 may be capable of further subdivision would not preclude its identity with Goldberger's pellagra-preventive "P. P." factor, itself possibly dual. In man two distinct conditions are described: pellagra, and *pellagra sine pellagra*, in which no skin eruption occurs. The "P. P." factor is effective against both.

In our experience the distribution of vitamin B_2 as determined by the method given above corresponds closely with that of the "P. P." factor and of the factor which prevents black-tongue in dogs, investigated by Goldberger, Voegtlin and others. The estimations made by these workers of the "P. P." factor contained in various foods are approximate, since in few cases was any attempt made to estimate the minimum necessary for protection, and the

basal diet was necessarily varied. Their results are collected in Table VI, the amounts given being expressed as the percentage of the dry weight of the diet, in order to obtain a rough comparison with our own results.

Table VI. *Comparison of the effects of various foodstuffs on vitamin B_2 deficiency in rats, on pellagra in man, and on black-tongue in dogs.*

Material	Rat Vitamin B_2	Man P.P. factor	Dog Black-tongue preventive factor
Maize endosperm	Very poor	Associated with pellagra in U.S.A. and Italy	—
Wheat flour	Very poor	Associated with pellagra in U.S.A.	Used in basal diet to produce the disease
Whole maize	Poor; 50 % less than normal growth	Traditionally associated with pellagra	No preventive action, used in basal diet to produce the disease
Whole wheat	Poor, but better than maize	—	66 % in diet delayed onset of the disease; no protection
Peas	30–40 % needed for good growth ("Clipper" peas)	30–35 % (168 g. daily); incomplete protection (Virginian cowpea)	58 %; slight protection, 73 %; almost complete protection (Virginian cowpea)
Wheat germ	15–30 % good growth	30 %; protection almost complete	31 %; protection almost complete
Fresh milk	5–10 % milk solids, good growth	20 % solids (1200 g. buttermilk daily) protected	250–300 cc. skim milk daily protected
Lean meat	7–10 % (dry wt.) good growth	10 % (dry wt., 50 g. daily) protected	11 % (dry wt.) protected
Dried yeast	1–3 % good growth	7 % (30 g. daily) protected	5 % protected
Liver	1–2 % (dry wt.) ox liver, good growth	Alcoholic extract of ox liver effective in treatment	11 % dried pork liver protected
Dried egg yolk	7–15 % good growth	—	17 % protected

Dried yeast is strongly preventive of pellagra; 30 g. daily averted the disease in asylum patients, otherwise liable [Goldberger and Tanner, 1925]. Fresh or autoclaved, it is also effective against black-tongue, if present in the proportion of 2–5 % of the basal diet [Goldberger *et al.*, 1928, 1].

Goldberger's original field studies in pellagra [Goldberger, Wheeler and Stucker, 1920] showed that "increasing supplies of milk or fresh meat were associated, one independently of the other, with a decreasing pellagra incidence," and later work has demonstrated the protective value of both foods against pellagra and against black-tongue [Goldberger *et al.*, 1924, 1926, 1928, 2]. Egg yolk, not yet tested in human pellagra, averts the canine disease [Goldberger *et al.*, 1928, 2]. Cowpeas gave some protection against both pellagra and black-tongue [Goldberger *et al.*, 1927, 1928, 2], but were not reckoned a good source of the protective factors.

An 85 % alcoholic extract of pork liver was used with good effect in the treatment of pellagra by Voegtlin [1920, Voegtlin *et al.*, 1920]. It was claimed

that a daily therapeutic dose equal to 1 kg. of fresh liver produced as good an effect as "a diet containing a considerable quantity of milk, eggs and meat." Dried pork liver is also preventive of black-tongue [Goldberger *et al.*, 1928, 2]. We found dried ox liver to be the most potent source of vitamin B_2 among the substances tested.

We found a mixed whole wheat, in which Manitoba wheat predominated, to have more vitamin B_2 potency than white or yellow maize. If vitamin B_2 and the "P. P." factor are identical, this result might explain the observed liability to pellagra of the maize eater rather than of the wheat eater, in localities where the whole grain and not highly milled products are consumed. Similarly, studies on black-tongue [Goldberger *et al.*, 1928, 1, 2] showed that the appearance of symptoms was earlier when the basal diet contained 78 % whole maize than when it contained 50 % whole wheat.

The endosperm of these two cereals, white flour and maize "grits," we found, on the other hand, to be about equally low in vitamin B_2 content. Therefore, if this vitamin be held to be a preventive of pellagra, white flour and maize endosperm should be equally conducive to the disease. This is possibly the case. Goldberger, Wheeler and Stucker [1920], in their field studies, found, when considering the poorest households with low supplies of fresh meat and milk, that no regular inverse ratio could be made out between the incidence of pellagra and the amount of white flour consumed. A rise in the household supply from 3 to 16 lbs. per adult male over a 15-day period did not cause any regular fall in the percentage of households affected by the disease. In the first report of the Thompson, McFadden Pellagra Commission [Siler, Garrison and MacNeal, 1913] it was stated that in the pellagra-affected districts studied, "wheat flour, in the form of bread or biscuit, is the principal bread-stuff, and corn-meal, while extensively used, is not nearly so staple an element of the dietary as wheat flour." It is possible that, where it is the custom to eat highly milled cereals, as in the United States, the more frequent association of pellagra with a maize than with a wheat diet is accidental, depending on other deficiencies in the diet of the maize eater. A maize diet is usually associated with poverty and marked by a shortage of such expensive foodstuffs as milk, meat and eggs.

Pellagra seems to be a disease of recent origin in the United States. According to Wood [1909], only sporadic cases were reported before 1909, and modern methods of milling are often held to be responsible for its recent increased prevalence. Our results support this to some extent, at any rate as far as wheat is concerned, since of all the cereal products examined we found wheat bran and wheat embryo richest in vitamin B_2.

Here again our results conform with those of Goldberger and his colleagues [1927, 1928, 2] on pellagra and black-tongue. A diet containing 60 % of maize germ meal cured two rats suffering from severe dermatitis, but since only poor growth occurred with a diet containing 30 %, maize germ meal must be considered a low source of the vitamin. Wood [1920] claimed to have fed

pellagrins on maize germ with prompt improvement. In our own experiments whole yellow maize as source of vitamin B_2 gave better growth than yellow maize "grits" (endosperm), showing that vitamin B_2 is lost in milling. This is more definitely the case with wheat, and it may be that the onset of pellagra in the United States coincided with the introduction of standard highly milled wheat and maize flour.

Evidence of the identity of vitamin B_2 deficiency in rats, of pellagra in man, and of black-tongue in dogs, is given in Table VI. The substances tested for the pellagra-preventive factor by Goldberger and his colleagues were given as daily additions to a daily diet of 400–500 g. (dry weight), equal to 2000–2500 calories. In the black-tongue experiments the test substances were incorporated in dietary mixtures of 400–500 g. (dry weight). From the tables given by these workers the percentages of the test substances in the diet have been roughly calculated.

There is very fair agreement in the effects of the various foods on the three conditions, and there seems little doubt that the condition produced in rats by a diet deficient in vitamin B_2 is the analogue of human pellagra. We suggest that our method might be useful in tackling any problems in the human disease that remain to be solved.

It is worth noticing that the rat, as compared with man and the dog, is relatively insusceptible to pellagra. Diets that produce florid pellagra in man and black-tongue in dogs do not cause dermatitis in rats, and only a rigorous purification of the basal diet can ensure the appearance of symptoms.

Summary.

(1) The method previously described by Chick and Roscoe [1928] has been used for estimating the vitamin B_2 value of foodstuffs. The special features of this method are (*a*) elaborate purification of the caseinogen in the basal diet, and (*b*) the use of Peters's antineuritic concentrate as source of vitamin B_1.

(2) The vitamin B_2 value of a substance was estimated by the minimum amount required to support normal increase in body-weight (11–14 g. per week) in young newly weaned rats receiving, apart from the test substance, a diet complete in all respects except vitamin B_2. For one week previous to the test the rats were maintained on a diet entirely free of both the B vitamins.

(3) Control rats from each litter used were fed on the basal diet, complete except for vitamin B_2. All remained stationary in weight and the majority developed skin symptoms.

(4) Some curative experiments on such rats suffering from dermatitis were carried out with materials containing vitamin B_2. In these amelioration of symptoms and resumption of growth were parallel.

(5) The vitamin B_2 value of wheat and maize is poor, that of maize being on the whole the lower in the samples examined. In wheat the germ and bran are better sources than the endosperm, and about equal to each other. In maize the germ is not so rich a source, but whole maize is better than maize endosperm.

(6) Dried peas have a low vitamin B_2 content.

(7) Dried ox liver, yeast and fresh whole milk are excellent sources of vitamin B_2. Dried meat and egg yolk are less good but richer than cereals.

(8) The relation of vitamin B_2 to Goldberger's "P. P." factor and to the factor preventing black-tongue in dogs is discussed. It is noted that so far the distribution of all three has been found to be identical.

(9) Some problems in the epidemiology of pellagra are discussed on the assumption that vitamin B_2 is identical with Goldberger's pellagra-preventive factor.

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LVI. A METHOD FOR THE ASSAY OF THE ANTINEURITIC VITAMIN B_1, IN WHICH THE GROWTH OF YOUNG RATS IS USED AS A CRITERION.

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The antineuritic vitamin (vitamin B_1 in the nomenclature provisionally adopted by the Biochemical Society) was discovered in 1897 by Eijkman [1897] and was recognised by its ability to prevent and cure polyneuritis which developed in birds fed upon a restricted diet. The general distribution of this vitamin in natural foodstuffs was worked out by Eijkman and his colleague Grijns [1901] and quantitative assay was later attempted by Cooper [1913, 1914] and by Chick and Hume [1917,1, 2] by comparison of the minimal doses required to prevent (or cure) the polyneuritis of pigeons fed upon a diet of polished rice.

The antineuritic B_1 vitamin was formerly believed to be identical with, and is now known to be one constituent of, the complex "water-soluble B" discovered by McCollum and Davis [1915, 1] and found by them to be necessary in a diet to ensure normal growth in young rats.

It is now certain [Goldberger *et al.*, 1926; Chick and Roscoe, 1927] that the antineuritic vitamin B_1, equally with the second identified constituent of "water-soluble B," viz. the heat-stable, antidermatitis, (?) antipellagra, vitamin B_2, is necessary for normal growth of young mammals. Young rats just weaned are so sensitive to absence of vitamin B_1 that they seldom survive more than 3–4 weeks even if vitamin B_2 is provided, death usually taking place from inanition without symptoms of paralysis. If, however, the antineuritic vitamin is administered before the condition of the animal is too far advanced recovery takes place and normal growth is established. In rats the suddenness of death and the inconstancy of the symptoms following vitamin B_1 deficiency make it impossible to assay this vitamin by any method based upon cure or recovery in the acute stage. But if foodstuffs containing this vitamin are administered as soon as the animal's weight has become stationary the increase in body weight which follows can be used as a criterion for estimating the content of vitamin B_1 in the material fed.

The present method for assay of this vitamin has been developed along the lines of that previously described for the assay of vitamin B_2 [Chick and Roscoe, 1928] and used by Aykroyd and Roscoe [1929] in their study of the distribution of this vitamin. In that method the provision of vitamin B_1 unaccompanied by vitamin B_2 is conveniently arranged by administering

Peters's antineuritic concentrate prepared from yeast. But in the analogous method for assay of vitamin B_1 the provision of vitamin B_2 unaccompanied by the former is not so easy. We have used washed brewery yeast, autoclaved at 120° for 5 hours, which retains about one-half the vitamin B_2 potency of the original yeast and is at the same time practically devoid of vitamin B_1. Sometimes, however, traces of vitamin B_1 appear to survive this treatment, the result, doubtless, depending on the amount of vitamin B_1 contained in the original yeast. Another drawback to the use of autoclaved yeast is its tendency to cause diarrhoea. Further heating to destroy all traces of vitamin B_1 is not advisable, for the amount of the deleterious substances would thereby be increased, and as more vitamin B_2 would also be destroyed, larger doses would be required.

The occurrence of diarrhoea complicates experiments with B vitamins for two reasons. If severe, the animal naturally suffers apart from any dietetic deficiency; if slight, the loose stools passed do not fall cleanly through the meshed wire floor of the cage [Chick and Roscoe, 1928, p. 796] and more opportunity is afforded for faeces-eating, which is a common habit among rats deprived of B vitamins and tends to mitigate the effects of the deficiency [Steenbock, Sell and Nelson, 1923; Kennedy and Palmer, 1928].

A more convenient, but, for reasons stated below, less satisfactory, source of vitamin B_2 free from vitamin B_1 was found in the fresh white of hen's eggs, a surprising fact, since the yolk contains both factors in abundance. One can only conclude that the yolk membrane is impervious to the passage of vitamin B_1.

A basal diet (E.L. diet) was prepared, in which the purified caseinogen of our usual "-B" diet (P_2L diet) was replaced by egg-white (see Table I). The relative amounts of dry constituents were the same as in the P_2L diet, the protein amounting to 20 % in either case. In preparing the diet the egg-white is separated from fresh eggs and coagulated by heating in a double saucepan containing boiling water, put through a mincing machine and then thoroughly mixed with the dry constituents of the diet, the small extra amount of water being worked in afterwards. The prepared diet is steamed for 3 hours at 100° in order to cook the starch thoroughly and prevent the risk of "refection" [Fridericia *et al.* 1928; Roscoe, 1928].

Table I. *Basal diets used in the study of the (antineuritic) vitamin* B_1.

E.L. (egg-white) diet. (g.)			P_2L (caseinogen) diet. (g.)	
Egg-white 800.	Protein	100		
	Water	700		
Rice starch		300	Purified caseinogen	100
Cotton seed oil		75	Rice starch	300
Salt mixture (McCollum's 185)		25	Cotton seed oil	75
Distilled water		50	Salt mixture (McCollum's 185)	25
		1250	Distilled water	500
				1000

0·4 g. autoclaved yeast daily was given to rats on diet P_2L to provide vitamin B_2. Cod-liver oil, to supply vitamins A and D, was administered to all rats separately by hand, 3 to 5 drops (0·05 g. to 0·1 g.) according to the size.

Young rats (40–50 g. weight), receiving the egg-white diet shortly after weaning, usually continued to grow for 2–3 weeks, after which the weight declined for lack of vitamin B_1. Death occurred if this was unchecked, but if Peters's antineuritic concentrate were given (0·1 cc. daily, equivalent to 0·6 g. dry yeast), the animals recovered and began to grow at the normal rate. In many cases they continued to grow normally for several weeks but a check was usually observed sooner or later before maturity was reached.

With young rats on the P_2L (caseinogen) diet supplemented with 0·4 g. autoclaved yeast daily to provide vitamin B_2, the preliminary period of growth generally lasted a week or two longer, owing possibly to traces of undestroyed vitamin B_1, which had survived the strong heating. In some

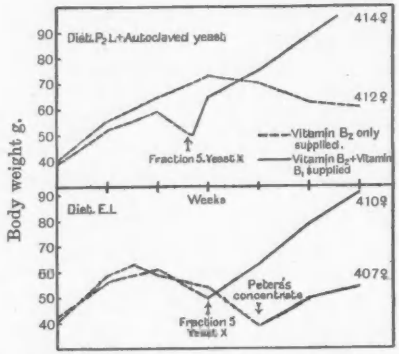

Fig. 1. Failure to grow of young rats from Litter 1184 on diets deprived of the antineuritic vitamin B_1, but containing egg-white (diet E.L.) and autoclaved yeast, respectively, as sources of vitamin B_2.

Rats 414 and 410 restored by equal doses of the same vitamin B_1-containing yeast extract; rat 407, by Peters's antineuritic concentrate.

instances the degree of growth displayed later during recovery, during the test of the vitamin B_1-containing material, was also greater than in rats receiving the egg-white diet. But in many cases concordant results were obtained when two rats of the same sex and litter, after suitable preparation on the two respective diets, received the same dose of vitamin B_1 (see Table II). This is illustrated by the weight curves of the four rats shown in Fig. 1. Two rats, 407 and 410, received E.L. diet and began to decline in weight after 2 weeks. After 4 weeks 407 was *in extremis* but was revived by a daily dose of Peters's antineuritic concentrate. Of the two rats on diet P_2L with a daily addition of 0·4 g. autoclaved yeast, 414 also grew for 2 weeks and then rapidly lost weight, but rat 412 was more resistant and attained the weight of 73 g. on this diet and after 6 weeks showed no sign of vitamin B_1 deficiency, although the body weight (68 g.) was then slowly falling.

Rats 414 and 410, after $2\frac{1}{2}$ weeks and 3 weeks on their respective diets deprived of vitamin B_1, were treated with the same dose of a yeast fraction

containing this vitamin. The degree of growth induced was the same in both cases (see also Table II, Exp. 1).

In Table II are collected the results of a series of tests in which similar doses of vitamin B_1-containing materials were administered to pairs of rats prepared respectively on the two different basal diets. In series (*a*) the results are fairly concordant, in series (*b*) the rats receiving autoclaved yeast grew somewhat better than those on the egg-white diet.

It is hoped to make a survey of the antineuritic (vitamin B_1) content of some of the commoner foodstuffs for comparison (*a*) with that already made, using prevention and cure of polyneuritis of birds as a criterion, and (*b*) with the distribution of vitamin B_2 already published by Aykroyd and Roscoe [1929]. If the unit of vitamin B_1 be defined as the dose required to restore normal (11–14 g. weekly) growth to a young rat, of which the growth has failed on a basal diet deficient only in vitamin B_1 and containing excess of vitamin B_2, the assay of a foodstuff consists in determining the minimal dose necessary for this degree of restoration.

Table II. *Comparison of (antineuritic) vitamin B_1 assay using basal diets containing egg-white (diet E.L.) and autoclaved yeast (diet P.Y.) respectively as sources of vitamin B_2.*

Series	Exp.	Material	Daily dose	Litter	Rat	Body wt, g.	Diet	Weekly increase in wt, g.	Av.
(*a*)	1	Yeast Fraction X_5	Equiv. to 0·12 g. dry yeast	1184	410 ♀	50	E.L.	13, 16, 12	14
				,,	414 ♀	51	P.Y.	17, 12, 16	15
	2	,, XI_2C	,, 0·25 ,,	1173	397 ♀	59	E.L.	9, 14	11·5
				1184	406 ♂	61	P.Y.	11, 14	12·5
	3	,, XII C	,, 0·25 ,,	1184	409 ♀	45	E.L.	21, 13, 14	16
				1173	396 ♀	39	P.Y.	16, 13, 7, 10	11·5
	4	,, XII C*a*	,, 1·0 ,,	1118	373 ♀	49	E.L.	22	22
				1117	361 ♂	43	P.Y.	23, 34	28
	5	,, XII_5	,, 0·12 ,,	1118	368 ♂	51	E.L.	20, 17, 11, 15, 14	15
				1117	360 ♂	40	P.Y.	18, 17, 7, 17, 11	14
(*b*)	6	,, XII_5C	,, 0·25 ,,	1291	462 ♂	46	E.L.	15, 18, 20, 21	18·5
				1298	470 ♂	41	,,	17, 13, 18, 15	16
				1291	459 ♂	61	P.Y.	26, 21, 18, 13	19·5
				1298	468 ♂	54	,,	28, 10, 18, 15	18
	7	Dried egg yolk	0·5 g. = 1·9 fresh	1291	464 ♀	40	E.L.	2, 2, 1	2
				,,	461 ♀	51	P.Y.	7, 3, −1	3
		,,	1·0 g. = 3·9 fresh	1291	465 ♀	38	E.L.	16, 18, 13, 8	14
				1298	466 ♂	72	P.Y.	26, 19, 12, 9	18·5

The basal diet containing egg-white as source of vitamin B_2 would be in every way the more convenient of the two diets described, were it not that this diet, even when supplemented with adequate vitamin B_1, as Peters's antineuritic concentrate, does not maintain growth of rats to maturity. That egg-white contains in abundance the antidermatitis vitamin B_2 is shown by the many satisfactory cures of the "pellagrous" condition which have been obtained with comparatively small doses [Aykroyd and Roscoe, 1929]. It would seem that some third factor in the vitamin B complex, other than vitamins B_1 and B_2, is present in autoclaved yeast, and lacking in egg-white.

The heat stability of this hypothetical third substance is an argument against its identity with those suggested by the work of Williams and Waterman [1927, 1928] and of Reader [1928].

The matter is being investigated further. So far our observations do not permit a definite conclusion to be drawn upon the extent to which the absence of this hypothetical third factor may affect the validity of the test for vitamin B_1 described in this paper. In a few instances the growth of rats has become subnormal in the first few weeks after weaning, but it has usually remained unaffected until at least 6–10 weeks after weaning (about 9–13 weeks from birth), by which time the animal has attained a body weight of 100–130 g. No symptoms of ill health have been found in rats observed for three to four months after growth has become sub-normal. Nevertheless, pending the results of further investigation it seems safer, in the assay of vitamin B_1, to use autoclaved yeast as the source of vitamin B_2 in the diet rather than egg-white.

Summary.

1. Methods are described for assay of the antineuritic vitamin B_1, using growth of young rats as a criterion and basal diets in which the more heat-stable vitamin B_2 is supplied by fresh egg-white or by yeast autoclaved at 120° for 5 hours.

2. Fresh egg-white, while rich in vitamin B_2, is devoid of vitamin B_1. With autoclaved yeast there is a risk that traces of vitamin B_1 which have escaped destruction in the autoclave may be present. Nevertheless autoclaved yeast is to be preferred to egg-white for the reason given in 3, below.

3. Diets otherwise complete, which contain egg-white and Peters's antineuritic concentrate as sources of vitamins B_2 and B_1 respectively, are frequently found unable to maintain normal growth of rats beyond a few weeks after weaning. After this period growth is subnormal. It is suggested that a third hypothetical dietary factor may be present in the vitamin B complex, which factor is heat-stable, present in autoclaved yeast and lacking in egg-white.

4. If the unit of vitamin B_1 be defined as the amount which will restore normal growth (weekly increase in weight of about 10–14 g.) in young rats whose growth has failed on a basal diet deficient only in this vitamin, assay of a foodstuff for content of vitamin B_1 would consist in determination of the minimum dose necessary to restore this degree of growth.

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LVII. AN ATTEMPT TO SEPARATE VITAMIN B_2 FROM VITAMIN B_1 IN YEAST AND A COMPARISON OF ITS PROPERTIES WITH THOSE OF THE ANTINEURITIC VITAMIN B_1.

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Yeast contains both constituents of the "water-soluble B" vitamin, vitamins B_1 and B_2, in abundance, and seeing that the antineuritic (vitamin B_1) concentrate prepared by the method of Peters [1924] is free from vitamin B_2 [Chick and Roscoe, 1927] our first endeavour towards the separation of this vitamin was to scrutinise every stage of the process to find where the disappearance occurred.

Our method has been that of Kinnersley and Peters [1925, 1927] with a few modifications. 10–12 kg. of pressed, brewery yeast were washed three or four times with cold tap water. The pressed yeast was puddled with water to the consistency of cream, filtered on a Gooch filter, using slight suction, and finally sucked dry or pressed in a hand press, this process being repeated three or four times until the filtrate was of a pale straw colour. After removal of a small sample for determination of the dry weight, the washed, pressed yeast (in the proportion of 1 kg. pressed, or 200 g. dry, yeast to 2–3 litres water) was thrown into boiling tap water containing 0·02 % acetic acid; this was raised again to the boiling point and allowed to boil for 5 minutes and filtered. This extract ("Fraction 5") about 20–30 litres in volume, was then treated with 25 % lead acetate solution till no more precipitate was formed (*ca.* 55–80 cc. per litre of extract), the whole allowed to stand overnight and then filtered. To the filtrate ("Fraction 4") baryta, at first solid and then as saturated solution, was added, till no more precipitate formed. As the resulting solution is alkaline, filtration was carried out at once and the filtrate acidified with strong H_2SO_4 until a p_H of about 4·0 was reached (using the indicator bromocresol green or Congo red). The solution, heated to assist agglutination of the precipitated $BaSO_4$, was filtered, and to the filtrate Hopkins's reagent (10 % solution of $HgSO_4$ in water containing 70 cc. H_2SO_4 per litre) was added to the point of most complete precipitation (25–50 cc. per litre of filtrate). In order to remove any traces of lead and mercury that might be present, H_2S was passed through the filtrate to saturation and the whole allowed to stand several hours in corked bottles; on heating the solution,

the metallic sulphides settled well and could readily be filtered. If the previous precipitation with baryta is omitted, these sulphides tend to remain in the colloidal form and are separated with difficulty. The reaction of the filtrate was then carefully adjusted to the neutral point (using litmus or phenol red) and treated twice with norite charcoal which had been purified before use by boiling with dilute hydrochloric acid and thorough washing with distilled water to remove all traces of acid. The charcoal was filtered off, washed and sucked dry on a Gooch filter and the filtrate treated with a second quantity. 60–70 g. norite per kg. dry yeast were used in each adsorption. Both portions of charcoal were boiled two or three times with fresh quantities of dilute acid alcohol (50 cc. alcohol, 50 cc. water, 1 cc. concentrated hydrochloric acid) and the combined extracts reduced to a convenient bulk (the equivalent of 6 g. yeast contained in 1 cc.) by distilling under reduced pressure at a temperature of 40 to 50°.

After each precipitation a portion of the filtrate was set aside and examined by the method already published [Chick and Roscoe, 1928] for its content of vitamin B_2. It is necessary to work quantitatively, determining in each case the minimum dose required to maintain a standard degree of growth (a weekly increase in body weight of 11–14 g.) and referring the dose given to the equivalent amount of the original dried yeast.

Table I. *Details of preparation from Yeast V of Peters's antineuritic vitamin B_1 concentrate, and results of tests of filtrates at each stage for content of vitamin B_2*.*

Process	Material tested	Dose given, expressed as equivalent of dry yeast g.	No. of rats observed	Average growth g. per week
	Dried yeast (from Table III, Chick and Roscoe [1927])	0·2	—	11
		0·4	—	23
1. Extraction of pressed, washed Yeast V with boiling water containing 0·01 % acetic acid	*Fraction* 5. Filtrate from 1 after evaporation to small bulk	0·25	3	12
		0·5	3	21
		1·0	3	29
2. Precipitation with Pb acetate at p_H 4·7 (800 cc. 25 % Pb acetate soln. per kg. dry yeast)	*Fraction* 4. Filtrate from 2 after evaporation to small bulk and removal of Pb with H_2SO_4	1·0	2	14
3. Precipitation with baryta and acidification with H_2SO_4 to p_H *ca.* 4·0 to precipitate $BaSO_4$	*Fraction* 3. Filtrate from 3 after evaporation and partial neutralisation with removal of any further traces of $BaSO_4$	2·0	2	6
		3·4	2	15†
4. Precipitation with acid mercuric sulphate, Hopkins's reagent (260 cc. per kg. dry yeast), and removal of traces of Hg by H_2S	*Fraction* 2. Filtrate from 4 after evaporation to small bulk	1·0	1	0
		2·0	1	0
		3·0	1	2
5. Adsorption of vitamin B_1 by treatment with norite (100 g. norite per kg. dry yeast, in two portions) after neutralisation	*Fraction* 1. Filtrate from norite after evaporation to small bulk	4·0	2	2·5

* Method of Chick and Roscoe [1928]. Growth of young rats observed for 2–4 weeks on a diet deprived of B_2 vitamins, but receiving vitamin B_1 as 0·1 cc. (=0·6 g. yeast) Peters's antineuritic concentrate.

† A similar fraction prepared from yeast VII was used for part of this test. These tests followed on those of the 2·0 g doses.

In Table I are set out the details of one such preparation, viz. from Yeast V. There was little loss of vitamin B_2 in Fraction 5, the first extract

from the yeast with acidified boiling water. The average weekly growth-increments of young rats receiving daily doses equivalent to 0·25 g. dried yeast were 12 g., about the same as that of rats receiving 0·2 g. daily of dried yeast; daily doses equivalent to 0·5 g. dried yeast had about the same effect as 0·4 g. dried yeast.

During precipitation with lead acetate there was a loss. Daily doses of the filtrate, Fraction 4, equivalent to 1·0 g. dry yeast produced an average weekly increase in weight of only 14 g. in two male rats, whereas an equivalent amount of Fraction 5 induced about twice as much growth (29 g.).

The precipitation with baryta caused a further loss in vitamin B_2 and, after precipitation with mercuric sulphate and subsequent removal of traces of mercury from the filtrate with SH_2, the filtrate contained no significant amount of this vitamin. Doses equivalent to 2 or 3 g. of the original dried yeast did not produce more growth than occurred in some control animals on the basal diet.

One may, therefore, conclude that about one half to three-fourths of the vitamin B_2 contained in the original yeast is carried down with the lead acetate precipitate; of the remainder, the greater part (about two-thirds) disappears with the precipitate formed by baryta, and the rest with that given with acid mercuric sulphate. Attention was, therefore, directed to the precipitation with lead acetate.

Influence of hydrogen ion concentration on the amount of vitamin B_2 carried down by lead acetate.

In the Peters process as described above, the precipitation takes place at a p_H of about 4·5–4·7 (bromocresol green). We tried precipitation at p_H 2·6 (bromophenol blue), this being the degree of acidity attained during the precipitation with Hopkins's reagent in the above process, when the last remaining traces of vitamin B_2 were found to be adsorbed. Precipitation with lead acetate at p_H 6·3–8·9 (using bromocresol purple and phenol red as indicators) was also tried.

At p_H 2·6 less than one-half the vitamin B_2 present was removed by lead acetate, at p_H 4·7 about three-fourths. In neutral or slightly alkaline solution the experiments indicated that all was carried down. Whereas of the original extracts the equivalent of 0·25 g. to 0·5 g. dry yeast, respectively, supported normal growth, little or no growth was induced by doses of the filtrates equivalent to 1·0 or 2·0 g. dry yeast (for details see Table II, Fractions 4, Yeast VIII and Yeast X). Unfortunately, as will emerge later, none of these precipitates with lead acetate was consistently free from vitamin B_1.

Recovery of vitamin B_2 from the lead acetate precipitate.

Vitamin B_2 can be recovered by decomposing the lead acetate precipitate with sulphuretted hydrogen. If the reaction is acid, however, the lead sulphide remains in colloidal solution, owing to the action of the yeast gum which

possesses an alkaline isoelectric point. If the solution is brought to p_H 8–9 (thymol blue) the lead sulphide separates readily, but in that case vitamin B_2 is adsorbed on the precipitate and the solution correspondingly impoverished. To prevent loss in this way the reaction should not be less acid than p_H 3·0 (bromophenol blue). The technical trouble caused by the yeast gum at a higher p_H can be overcome by hydrolysing it in the original extract.

Table II. *Influence of hydrogen ion concentration upon the removal of vitamin B_2 (and B_1) from an extract of brewers' yeast by precipitation with lead acetate.*

Estimation of vitamins B_2 and B_1 in the filtrates and in the solutions obtained by decomposition of the precipitate by methods of Chick and Roscoe [1928] and [1929] respectively.

Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Av. growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Av. growth g. per week	Dose (expressed as equivalent in g. of dry yeast) required to give normal (11–14 g. weekly) increase in weight as source of: Vitamin B_2	Vitamin B_1
Yeast VIII:							
Fraction 5. Extract with dilute acetic acid	0·12	2	7	—	—	0·25	—
	0·25	4	11	—	—		—
	0·5	1	18	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 2·6	0·5	3	13	—	—	0·5	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 4·7	1·0	1	13	—	—	1·0	—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 6·3	0·5	1	0	—	—	No growth with 1·0	—
	1·0	1	0	—	—		—
Fraction 4. Filtrate after precipitation of Fraction 5 with Pb acetate at p_H 8·9	1·0	3	0	—	—	,,	—
Yeast X:							
Fraction 5	0·25	4	9	—	—	0·25 to 0·5	—
Fraction 5, after hydrolysis 1 hour 100° at p_H 1·5	0·5	3	13	—	—		—
	0·25	1	13	—	—		—
Fraction 4. Filtrate after precipitation with Pb acetate at p_H 7·2	1·0	1	7	—	—	>2·0	—
	2·0	3	4	—	—		—
Fraction C_1 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 4·7	0·5	1	10	1	1	0·5 to 1·0	>2·0
	1·0	1	14	1	0·2		
	2·0	—	—	1	0·3		
Fraction C_2 by decomposition with SH_2 of precipitate formed with Pb acetate at p_H 7·5	0·5	3	11	1	7·5	0·5 to 1·0	0·5 to 1·0
	1·0	1	14	1	14		
Yeast XI:							
Fraction 5	0·25	2	11	—	—	0·25	—
Fraction C_1, from Pb acetate precipitate formed at p_H 4·7	0·25	2	11	2	12	0·25	0·25
	0·5	2	9	1	18		
	1·0	1	19	—	—		
Fraction C_2, from Pb acetate precipitate formed at p_H 7·5	0·5	2	9	—	—	0·5 to 1·0	<1·0
	1·0	1	15	1	25		
Yeast XII:							
Fraction 5	0·12	—	—	2	15	0·25 to 0·4	0·12
	0·25	2	8·5	—	—		
	0·4	2	14	—	—		
Fraction C_2, from Pb acetate precipitate formed at p_H 7·3	0·25	—	—	4	15	0·5	0·25
	0·5	6	14·5	1	20		

The following procedure was adopted. To 1 litre of the original dilute acetic acid yeast extract (Fraction 5) were added 10 cc. concentrated hydrochloric acid, making the p_H about 1·5 (thymol blue). The mixture was heated in steam at 100° for 1 hour, neutralised, the p_H adjusted to about

7·5 (phenol red) and 10 % solution of basic lead acetate added till precipitation was complete. The liquor was decanted, the precipitate drained on a Gooch filter and washed with a small amount of distilled water. If not wanted immediately the precipitate can be dried and worked up later, but if kept in the moist condition it is liable to become infected with moulds. The precipitate was suspended in water, the reaction adjusted to p_H 3·0 (bromophenol blue), sulphuretted hydrogen passed to saturation and the whole shaken for about 2 hours. After standing, a clear yellow fluid should separate from the lead sulphide. This was filtered, the residue washed, again suspended in water and treated with sulphuretted hydrogen, the operation being repeated two or three times until the top fluid was colourless. The combined filtrates and washings, which were strongly acid, were neutralised with sodium hydroxide or baryta (preferably the latter) to *ca.* p_H 3·0 and evaporated on a water-bath to a convenient bulk (1–2 cc. containing the equivalent of 1 g. yeast), during which a small additional amount of lead sulphide was often deposited. This degree of heating does not seem to impair the activity of the preparation. The sodium sulphate present in this final product, referred to as Fraction C for brevity, sometimes caused diarrhoea in the doses administered to the rats; it can, however, be replaced by sodium chloride if precipitated by barium chloride, but care must be taken to avoid any excess.

Table II gives the details of such preparations made from Yeasts X, XI and XII respectively. In addition to the tests for vitamin B_2, the products, Fractions C, were also tested for content of vitamin B_2, using the method described in the accompanying paper [Chick and Roscoe, 1929].

The Fractions C_2, obtained from Yeasts X and XI by decomposition of the lead acetate precipitates formed at p_H 7·3 to 7·5, contained about one-half of the vitamin B_2 present in the respective original yeast extracts (Fractions 5). Fraction C_2, obtained in the same manner from Yeast XII, contained nearly the whole of the vitamin B_2 of the corresponding Fraction 5; normal growth was obtained with the equivalent of 0·5 g. yeast, the dry weight of the dose (less ash) being 0·03 g. It was, however, admixed with vitamin B_1 and although it contained only about one-half of that present in the original Fraction 5, it was richer in this vitamin than in vitamin B_2.

By precipitation with lead acetate in neutral or alkaline solution more vitamin B_1 is carried down than in the weakly acid solution (p_H 4·7) of Peters's original process. It was hoped that by precipitation at p_H 4·7 a method of separation would be obtained. From Yeast X a Fraction C_1, almost free from vitamin B_1 was indeed obtained from the lead acetate precipitate formed at this p_H. However, this was not found to occur uniformly (compare Fraction C_1 prepared from Yeast X with Fraction C_1 from Yeast XI, Table II). The reason for the discrepancy is not obvious but may be due to differences in the relative proportions of the two vitamins contained in the original yeast extract.

It is possible that by working throughout in more acid solutions, at p_H 2·0

to 3·0, a preparation of vitamin B_2 free from B_1 might be obtained, but it would be very weak. In the experiments made with Yeast VIII (Table II), when the lead acetate precipitation occurred at a low p_H, the amount of vitamin B_2 adsorbed was much reduced.

Yeast extract seems to be an unfavourable medium for separation of these two vitamins by the type of process here employed, and it is possible that success might be attained by working with some other material. Rosedale [1927] used a method resembling Peters's for preparing an antineuritic concentrate from rice polishings. On decomposing the lead acetate precipitate with sulphuretted hydrogen, he obtained a substance which maintained health and weight in pigeons but had no curative effect on polyneuritis, although affording temporary relief by causing evacuation of the bowel. At the present time little is known, except by inference, of the relation of vitamin B_2 to the nutrition of the pigeon, but it is probable that Rosedale's preparation contained vitamin B_2 without admixture of vitamin B_1.

Other methods tried for separating vitamin B_2 from B_1 in yeast extracts.

The vitamin B_2 preparations described above could doubtless be freed from vitamin B_1 by heating at a high temperature, but the vitamin B_2 content would be reduced at the same time. The vitamin B_1 in yeast was destroyed and the vitamin B_2 reduced to about one-half the original amount by heating to 120° for 5 hours [Chick and Roscoe, 1927, Table III].

Our endeavour was to effect a separation by other means than heat, and to this end we tried the following: (1) dialysis, (2) solubility in strong alcohol and (3) ultra-violet light. None was successful, but as the work afforded some information as to the properties of these two vitamins it seems worth while to place it on record.

Dialysis.

Fraction 5 (acetic acid extract) from Yeast X, which contained both vitamins B_1 and B_2, was placed in a bag of cellophane (a cellulose membrane prepared from viscose) and dialysed against distilled water. The solutions were acidified to p_H about 3·0) and dialysis was carried on in a refrigerator to prevent putrefaction. After 4 days, the inside and outside liquors were tested for content of vitamins B_1 and B_2 respectively. No difference could be detected in the concentration of vitamin B_2 in the fluid inside and outside the membrane. Vitamin B_1 also passed freely through the membrane (see Table III, exp. 1).

Solubility in alcohol.

There are many facts in the literature indicating that the antineuritic (vitamin B_1) component of the complex "water-soluble B" is more readily soluble in strong alcohol than the more heat-stable (vitamin B_2) component.

Table III. *Influence of dialysis, ultra-violet irradiation, and alcohol upon vitamins B_1 and B_2 respectively contained in a dilute acetic extract (Fraction 5) from yeast.*

Methods of assay as in Tables I and II.

Exp.	Material	Dose given expressed as equivalent of dry yeast (g.)	Vitamin B_2 content: No. of rats observed	Vitamin B_2 content: Average growth g. per week	Vitamin B_1 content: No. of rats observed	Vitamin B_1 content: Average growth g. per week
1	*Dialysis:*					
	Yeast X, Fraction 5 (extract in dilute acetic acid)	0·25	4	9	—	—
		0·5	3	13	—	—
	Yeast X, Fraction 5. Dialysed: outside liquor	0·25	1	8	1	10
		0·5	1	12	1	14
	inside liquor	0·25	1	8	—	—
		0·5	1	15	—	—
2	*Effect of ultra-violet light:*					
	Yeast XII, Fraction 5	0·12	—	—	2	15
		0·25	2	9	—	—
		0·4	4	14	—	—
	Yeast XII, Fraction 5. Irradiated with ultra-violet light for 6 hours	0·12	—	—	2	11·5
		0·25	1	4	—	—
		0·5	1	8	—	—
	for 12 hours	0·12	—	—	2	7
		0·25	—	—	1	8
		0·5	1	4	—	—
		0·75	1	9	—	—
3	*Solubility in alcohol:*					
	Yeast XII, Fraction 5. See above exp. 2.					
	Yeast XII, Fraction 5, after drying and exposure to air for 19 days	0·4	2	13·5	—	—
	Yeast XII, Fraction 5, after drying and exposure to 94 % alcohol for 19 days	0·4	2	11	—	—
	Yeast XII, Fraction 5. Fraction soluble in 92 % alcohol	0·25	—	—	2	12
		0·5	2	2	—	—
		1·0	2	0·5	—	—
	Yeast XII, Fraction 5. Fraction insoluble in 92 % alcohol	0·12	—	—	1	10
		0·25	—	—	1	16
		0·5	3	9	—	—

The antiberiberi, antineuritic vitamin, has long been known to be soluble in alcohol in strengths ranging from 88 % to absolute [Fraser and Stanton, 1910; Eijkman, 1911; Chamberlain and Vedder, 1911; Kinnersley and Peters, 1925, and others]. It was further observed by Schaumann [1911] that extracts made with 96 % alcohol from yeast or rice bran, while potent in the cure and prevention of polyneuritis, could not maintain the body weight of the experimental animals, whereas equivalent amounts of the original materials could do both. Drummond [1917] found it impossible to obtain a satisfactory extract of the complex formerly known as "water-soluble B" with absolute alcohol, but succeeded with 70 %. This discrepancy in alcohol-solubility between the antineuritic vitamin and the complex water-soluble B was one of the facts throwing doubt on the supposed identity of these two dietary factors [Mitchell, 1919].

After demonstrating the existence of the two vitamins in water-soluble B, Goldberger and his colleagues [1926] found an 85 % (by volume) alcoholic extract of white maize to be rich in the antineuritic vitamin B_1, but deficient

in the heat-stable P-P (vitamin B_2) component. They write, "we have gained the impression that 'P-P' is relatively much more soluble in acidulated water than in 85 % alcohol, whereas the antineuritic factor is soluble in both." In a recent paper Sherman and Sandels [1929] find vitamin B_2 ('vitamin G') to be insoluble in 95 % alcohol.

We have fully confirmed the conclusions of Goldberger and his colleagues and consistently find vitamin B_1 soluble, and vitamin B_2 insoluble, in alcohol of concentrations from 83 to 93 % by weight. But we have not been able to effect a satisfactory separation of the two vitamins in this manner. After the final recovery from the insoluble residue the vitamin B_2 was reduced in amount, suggesting that this vitamin was destroyed by contact with alcohol or by some other conditions experienced during the operation. Sherman and Sandels [1929], whose work appeared after these experiments were complete, came to a like conclusion.

The following illustrates the method used. Fraction 5, the dilute acetic acid extract from Yeast XII, was concentrated on a water-bath. Of the concentrate, 50 cc., equivalent to 82 g. yeast, were dropped slowly into $2\frac{1}{2}$ litres of 94 % (by weight) alcohol with vigorous stirring. A precipitate was formed immediately. The mixture was shaken for 2 hours and allowed to stand several days after which the clear yellow top liquor was siphoned off. The insoluble matter was filtered and, after washing, shaken with a fresh amount of 94 % alcohol and the operation repeated until the alcoholic extract was colourless.

The combined alcoholic extracts and washings, of which the specific gravity corresponded to strengths of alcohol varying from 91·5 to 93 % alcohol, were concentrated to a small bulk at a low temperature under reduced pressure and finally taken to dryness at 37° with a fan. This dry residue, which was very acid, and the dried insoluble precipitate (= 7·7 g.) were dissolved in water and separately tested for content of vitamins B_1 and B_2 respectively.

The results (Table III, exp. 3) showed that about one-half the vitamin B_1 present in the original material was in the alcohol-soluble fraction. Vitamin B_2 was not detected in this fraction, but the amount present in the alcohol-insoluble portion was only equal to about one-half of that originally present.

In order to see whether this destruction of vitamin B_2 were due to the contact with alcohol or to oxidation when in the dry condition, a sample of the concentrated yeast extract used for the above experiment was evaporated to complete dryness and portions were allowed to remain at room temperature exposed to the air, and covered with 94 % alcohol respectively. After 19 days the alcohol was blown off the latter at 37° with a fan and the dry residue taken up in water and tested for vitamin B_2 content. This was compared with that of both the original solution and the solution made from the air-dried material. The results, set out in Table III, exp. 3, showed that a slight diminution in potency had taken place in the material which had been in contact with alcohol, but the difference was not significant and seemed too small to account for the loss experienced in the experiment described above.

Further investigation is needed to elucidate this matter. It may be that the destructive effect of alcohol on vitamin B_2 depends on the reaction of the solution.

Action of ultra-violet light.

Hogan and Hunter [1928] reported experiments on growing rats in which "vitamin B" was supplied in the diet respectively by autoclaved yeast or yeast irradiated by ultra-violet light. From the results they concluded that the heat-labile antineuritic (vitamin B_1) component was resistant to the action of ultra-violet light, whereas the heat-stable component (vitamin B_2) was destroyed by irradiation. If this were so, an easy method would be available for preparing an antineuritic material free from vitamin B_2, which could replace Peters's concentrate in the study of vitamin B_2.

The action of ultra-violet light was tested upon the dilute acetic acid extract, Fraction 5, from Yeast XII, which is rich in both vitamins (Table III). The material was concentrated to a small volume (2 cc. containing the equivalent of 1 g. yeast) and poured over the surface of a flat white dish, to an average depth of about 2 mm. and irradiated at a distance of 40 cm. from a mercury vapour arc lamp. From time to time a little distilled water was added to prevent drying and the whole well mixed. After 6 hours' irradiation the content of vitamin B_2 was found to be reduced to about one-half, while that of vitamin B_1 had also suffered, though to a less extent. After 12 hours' irradiation, more than half the vitamin B_1 originally present had been destroyed. Further destruction of vitamin B_2 had also occurred, but this was by no means complete (see Table III, exp. 2).

Summary.

1. The Peters process, by which an antineuritic, vitamin B_1, concentrate free from vitamin B_2 is prepared from yeast, has been scrutinised to find out at which stage the removal of vitamin B_2 occurs.

2. About one-half to three-quarters of the vitamin B_2 present in the original yeast was carried down in the precipitation with lead acetate at p_H 4·7. The removal was more complete if the precipitation were carried out in neutral or slightly alkaline solution.

3. If the lead acetate precipitate is decomposed with sulphuretted hydrogen, a clear solution containing vitamin B_2 can be separated from the lead sulphide if certain precautions be taken.

The dose of this preparation required to maintain normal growth in young rats on diets deprived only of vitamin B_2, contained 0·03 g. dry weight (less ash) and was the equivalent of 0·5 g. of the original yeast.

4. This preparation contained more or less vitamin B_1, depending on the reaction at which precipitation with lead acetate had been carried out and probably on the amount (relative to vitamin B_2) contained in the original yeast.

5. Vitamin B_2 is insoluble, and vitamin B_1 is soluble, in alcohol of 92 % by weight. The method employed to effect a separation with strong alcohol destroyed vitamin B_2. The attempt to prepare a vitamin B_2 concentrate free from vitamin B_1, by the use of alcohol, was unsuccessful.

6. Both vitamins dialyse freely through cellophane (a viscose preparation of cellulose).

7. Ultra-violet light was found to exert a destructive action on both vitamins, vitamin B_2 being destroyed at a quicker rate than vitamin B_1. The result obtained with vitamin B_2 confirms the previous observation of Hogan and Hunter [1928]. Their conclusion that vitamin B_1 is resistant to ultra-violet light is not, however, confirmed.

In conclusion we desire to express our thanks to Sir Charles Martin for helping us by his continued advice and criticism.

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LVIII. THE EFFECT ON VITAMIN B_2 OF TREATMENT WITH NITROUS ACID.

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In 1918 McCollum and Simmonds reported that the dietary factor "water-soluble B" was resistant to the action of nitrous acid, the activity of the vitamin being tested by its power to restore growth in rats where increase in weight had failed on a diet deficient in this vitamin [1918]. Levene and Van der Hoeven [1926] confirmed this result. They found that the Osborne-Wakeman fraction prepared from yeast contained 90–100 % of its original "vitamin B" potency after deamination. Their tests also involved observations of the growth of rats [Levene and Van der Hoeven, 1924], but as the authors themselves admit [Levene, 1928], the time during which the rats were under observation (3–4 days) was too short for trustworthy results. McCollum and Simmonds observed the growth of their experimental animals over a period of 2 weeks.

Although the above experiments were made before the composite nature of the "water-soluble vitamin B" was recognised, it would be natural to infer that treatment with nitrous acid had not destroyed either of the two known components, vitamins B_1 and B_2, since both are necessary for growth. Peters [1924] found his antineuritic, vitamin B_1, concentrate prepared from yeast to be completely resistant to the action of nitrous acid as tested by its capacity, before and after treatment respectively, to cure polyneuritis in pigeons, but in a recent paper, Levene [1928] reports the opposite to be true of the heat-stable, antidermatitis, vitamin B_2. A vitamin B_1 concentrate, prepared by adsorption with silica gel from the Osborne-Wakeman fraction of yeast, was found to contain a small proportion of the heat-stable, vitamin B_2, factor, which could be removed by deamination. The activity of this vitamin is stated to be destroyed by treatment with nitrous acid, while the heat-labile (vitamin B_1 factor) remains intact. No description is given of the animal tests on which this statement is based.

The suggestion that the activity of vitamin B_2 might be dependent upon the presence of amino-nitrogen seemed important enough to be worth confirmation. Accordingly, the action of nitrous acid was tested on a vitamin B_2 concentrate prepared from yeast by decomposition with sulphuretted hydrogen of the precipitate formed when lead acetate is added to a dilute

acetic extract of washed brewery yeast [Chick and Roscoe, 1929]. This concentrate contained about 6 % of the dry weight, 3 % of the nitrogen and about one-half the vitamin B_2 of the original yeast. The daily dose required to maintain normal growth (weekly increase in weight of 11–14 g.) in young rats on a diet deprived only of vitamin B_2 was 0·03 g. (dry weight, less ash), and was contained in the equivalent of 0·5 g. of the original yeast. Vitamin B_1 was also present.

Deamination was carried out as follows, using the method described by Peters [1924]. To 50 cc. of the preparation (equivalent to 50 g. original dried yeast), which was already acid (p_H *ca.* 3·0), 50 cc. distilled water, 5 cc. of a 30 % solution of sodium nitrite and 10 cc. of 10 % sulphuric acid were added. The mixture was shaken by hand for 5 minutes, left to stand overnight, heated next day on a water-bath, and after all foaming had ceased, allowed to boil for 10 minutes. Estimations by Van Slyke's method (15 mins. shaking) gave 0·091 % amino-nitrogen (*i.e.* 0·091 g. per 100 g. of the original yeast) in the material before treatment, and this was reduced to about one-sixth, viz. to 0·016 % amino-nitrogen after treatment.

Table I. *Influence of deamination upon the vitamin B_2 activity of a preparation (XII_3C) obtained from brewer's yeast, daily dose 0·5 cc., equivalent to 0·5 g. dried yeast.*

The rats received diet P_3L deprived of B vitamins supplemented by daily doses of cod-liver oil (0·05–0·1 g.), to provide vitamins A and D, and of Peters's antineuritic concentrate (0·1 cc. equivalent of 0·6 g. yeast), to provide vitamin B_1.

Material	Nitrogen in preparation (reckoned per 100 g. of original yeast)		Litter No.	Rat No.	Body wt g.	Increase in body weight in successive weeks g.	Average	Mean
	Total	Amino						
Before treatment with nitrous acid	0·24	0·091	1264	451 ♀	36	15, 12, 13	13·3	14·0
			1285	452 ♂	41	16, 11, 15	14	
			,,	455 ♀	35	18, 14, 12	14·7	
After treatment with nitrous acid	0·13	0·016	1264	450 ♀	40	15, 14, 12	13·7	13·8
			1285	453 ♂	43	16, 17, 11	14·7	
			,,	454 ♀	37	12, 13, 14	13	

No diminution in the vitamin B_2 potency of the preparation could be detected. The results of the tests, using our method of vitamin B_2 assay [Chick and Roscoe, 1928], are set out in the accompanying Table I. Six young rats (35–43 g. weight) were divided into two groups, similar in respect of litter and sex, and maintained on a basal diet deprived of B vitamins with the addition of a daily dose (0·1 cc., equivalent to 0·6 g. yeast) of Peters's antineuritic concentrate, to provide vitamin B_1. One group of rats received the vitamin B_2 concentrate before treatment with nitrous acid (daily dose equivalent to 0·5 g. original dried yeast) and the second an equal dose of the material after deamination. Growth of both groups was observed for 3 weeks. The rats in the first group showed an average weekly increase in weight of 14·0 g. and those in the second of 13·8 g., proving that no destruction of vitamin B_2 had taken place during treatment with nitrous acid.

SUMMARY.

Levene's observation that the activity of vitamin B_2 is destroyed by the action of nitrous acid is not confirmed. A vitamin B_2 preparation from yeast was found to possess equal power after and before treatment to induce growth in young rats on a diet deprived of this vitamin.

My thanks are due to Miss M. H. Roscoe for her care of the experimental animals used in the tests.

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LIX. THE APPLICATION OF THE IODIMETRIC METHOD TO THE ESTIMATION OF SMALL AMOUNTS OF ALDOSES.

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One of the most interesting facts emerging from the study of the carbohydrate-phosphoric esters formed during alcoholic fermentation is the rapid conversion of glucose into derivatives of fructose and *vice versa*. The fermentation of either glucose or fructose by yeast juice, dried yeast or zymin may give rise to phosphoric esters of glucose, γ-fructose, trehalose and possibly of other sugars, in proportions which vary within wide limits according to the type of yeast and other conditions of the fermentation. For the investigation of these products a reliable method for distinguishing quantitatively between aldoses and ketoses is much to be desired.

The reaction between aldoses and alkaline solutions of iodine

$$R.CHO + I_2 + 3NaOH = R.COONa + 2NaI + 2H_2O$$

was first applied to the estimation of sugars by Romijn [1897] who, after comparative experiments, selected borax as the most suitable form of alkali. At 25° oxidation of glucose and other aldoses was found to be complete in 16–22 hours, but ketoses and non-reducing sugars were also oxidised to a varying extent. Bland and Lloyd [1914] employed a solution containing iodine and sodium hydroxide in equimolecular proportions, and claimed that, at room temperature, oxidation of glucose, maltose and lactose was complete in 5 minutes, while fructose and sucrose were not attacked. Bougault [1917] and Cajori [1922] used sodium carbonate as the alkali and allowed the reaction to proceed for 20–30 minutes at room temperature. Both emphasise the necessity of employing a large excess of iodine (three times the theoretical quantity). Colin and Liévin [1918] replaced the carbonate by disodium hydrogen phosphate without any obvious advantage. In Willstätter and Schudel's [1918] modification sodium hydroxide is used and is added last, in the proportion of 3 mols. to 2 mols. of iodine—as required by the equation. The oxidation is carried out at room temperature during 12–20 minutes. This modification has been most frequently employed in biochemical investigations but the authors' claim that under the specified conditions fructose and sucrose are not attacked has been disputed by Judd [1920] and Bruhns [1923], who have obtained values for fructose varying between 7 % and 11 %

in terms of the reducing power of glucose. Baker and Hulton [1920] found that oxidation of aldoses was complete in 3–5 minutes, but even under these conditions fructose was oxidised to the extent of 7 %. According to Judd this partial oxidation is the result of the Lobry de Bruyn and van Ekenstein transformation but the experimental evidence brought forward by Baker and Hulton does not bear out this suggestion.

The investigations of Kolthoff [1923] and of Hinton and Macara [1924] show that the degree of oxidation both of aldoses and of ketoses is influenced to a marked extent by the relative proportions of iodine and alkali, and that satisfactory results are only to be obtained by careful regulation of these and other factors. In a recent paper Goebel [1927] states that the oxidation of glucose is more complete if the alkali be added gradually over a period of several minutes. In all these investigations the estimations were carried out for the most part on relatively large amounts of the sugars. As we wished to apply the method to the estimation of quantities of the order of 1 mg. it seemed advisable to study first the effect of the various factors under the conditions involved in such micro-estimations.

The following is the general procedure adopted for these tests.

1 or 2 cc. of the sugar solution (0·1 %) was measured into a 50 cc. Erlenmeyer flask, followed by 3 cc. of 0·02 *N* iodine and distilled water to make up the volume to 6 cc., this last serving to rinse down the sides of the flask. The alkali solution was then added slowly and the flask immediately closed with a rubber stopper and immersed in a water-bath at the specified temperature. The use of "room temperature" was given up as the variations were found to be sufficient to produce irregular results. At the end of the given time the contents of the flask were acidified with 1 cc. 0·5 *N* H_2SO_4 and titrated with 0·005 *N* sodium thiosulphate using as indicator 3 drops of a 1 % solution of soluble starch in a saturated solution of sodium chloride. All estimations were made in duplicate and blank tests, also in duplicate, were simultaneously carried out, using distilled water in place of the sugar solutions. These blanks were further checked by direct titration of 3 cc. of the iodine solution with the thiosulphate. The difference between the blanks and the direct titration did not as a rule exceed 0·1 cc., and was due to loss of iodine by volatilisation, impurities in the alkali, etc. The usual precautions were taken as regards calibration of the Ostwald pipettes and the burette. The sugars used in these tests gave values for the specific rotation and reducing power by the Hagedorn-Jensen method as shown in Table I. The values for the reducing power are given in terms of glucose and for the other sugars are of limited significance, but the very low value for galactose, which is similar to that recorded by Pucher and Finch [1928], is of interest in relation to the results obtained by the iodimetric method.

The results given in Tables II and III show the influence of varying quantities of sodium hydroxide and sodium carbonate respectively on the oxidation of glucose and fructose.

Table I.

Sugar	$[\alpha]_{5461}^{20°}$ $c=10$ %	Reducing power %
Glucose (Kahlbaum)	+ 62·8° (21°)	99·9
Fructose (Kerfoot)	−108·7°	96·2
Galactose (Kahlbaum)	+ 96·9°	73·0
Lactose	+ 64·6° (19°)	67·0
Maltose hydrate (Kahlbaum)	+154·8°	68·5
Sucrose	+ 78·6°	—

Table II.

Alkali: 0·1 *N* NaOH, in amount varying from 1 to 1·66 times the equivalent of iodine.

Vol. of 0·1 *N* NaOH cc.	Temp.	Time (mins.)	Glucose (1 mg.)	Glucose (2 mg.)	Fructose (1 mg.)
0·6	17°	20	− 0·5	− 8·7	3·2
			− 0·5	− 8·7	3·2
0·75	,,	,,	− 0·5	−13·0	3·6
			− 1·0	− 8·7	3·6
0·9	,,	,,	+ 0·5	− 2·2	4·0
			+ 0·5	−10·0	4·0
1·0	,,	,,	+ 0·5	− 2·2	4·0
			0	− 5·7	4·0
0·6	21°	5	−15·7	—	0·4
			−15·7	—	1·2
0·75	,,	,,	−20·9	—	0·8
			−13·1	—	0·8
0·9	,,	,,	−12·1	—	0·8
			−13·1	—	0·8
1·0	,,	,,	−12·2	—	1·2
			−12·2	—	1·2
0·6	21°	20	− 0·5	−11·1	5·9
			+ 1·2	−11·1	7·5
0·75	,,	,,	− 0·5	−13·7	7·5
			− 0·5	−13·7	10·0
0·9	,,	,,	+ 0·9	− 2·4	8·8
			− 1·3	− 2·0	8·8
1·0	,,	,,	+ 0·4	− 1·3	9·2
			− 1·3	− 0·9	9·2

For glucose the results are stated as percentage deviations from the theoretical values for complete oxidation to gluconic acid.

For fructose the results are stated as the percentage oxidised, calculated on the basis of the theoretical value for glucose.

The amount of iodine used was in all cases 3 cc. 0·02 *N* solution, equivalent to 5·4 mg. glucose. The titration thus required (for the blank) about 12 cc. 0·005 *N* thiosulphate. 2·25 cc. is equivalent to 1 mg. glucose. The results, though calculated to the first decimal place, are only significant to about 0·5 %.

These results show that:

(1) With sodium hydroxide (0·9 cc.) in the proportion of 3 mols. to 2 mols. of iodine, as recommended by Willstätter and Schudel, the oxidation of 1 mg. glucose is complete in 20 minutes at 17° or 21°, but not in 5 minutes at 21° (iodine in 5-fold excess). With 2 mg. glucose the results for 17° are irregular but are more satisfactory for 20 minutes at 21°.

Table III.

Alkali: 5 % Na_2CO_3 solution (0·94 *N*).

Vol. of 5 % Na_2CO_3 cc.	Temp.	Time (mins.)		Glucose 1 mg.	Glucose 2 mg.	Glucose 3 mg.	Fructose 1 mg.	Fructose 2 mg.	Fructose 3 mg.
0·1	21°	30		+0·4	−23·1	−61·7	—	—	—
,,	,,	,,		0	−33·3	−57·5	—	—	—
,,	,,	,,		−4·1	—	—	—	—	—
,,	,,	,,		−9·8	—	—	—	—	—
			Av.	−4·5	−28·2	−59·6			
0·2	21°	30		+1·0	− 1·2	−18·5	0·4	0·8	1·4
,,	,,	,,		+1·0	− 1·2	−23·4	0·4	2·5	2·2
,,	,,	,,		−1·5	− 3·1	−33·3	1·7	—	—
,,	,,	,,		−2·4	− 3·1	−33·3	0·9	—	—
,,	,,	,,		−0·2	—	—	—	—	—
,,	,,	,,		−0·2	− 2·2	—	—	—	—
,,	,,	,,		−0·2	—	—	—	—	—
,,	,,	,,		−0·2	− 2·2	—	—	—	—
,,	,,	,,		+2·3	—	—	—	—	—
,,	,,	,,		+2·3	—	—	—	—	—
,,	,,	,,		+2·6	—	—	—	—	—
,,	,,	,,		+2·6	—	—	—	—	—
			Av.	+0·6	− 2·2	−27·1	0·8	1·6	1·8
0·4	21°	30		+0·7	− 1·7	−10·3	0·4	0	—
,,	,,	,,		+0·7	—	—	0·4	0	—
,,	,,	,,		+0·7	− 3·0	− 8·4	0	—	—
,,	,,	,,		−2·0	—	—	0	—	—
,,	,,	,,		−1·5	− 1·8	−11·6	0·4	—	—
,,	,,	,,		+0·3	—	—	—	—	—
,,	,,	,,		+0·7	− 0·7	—	0·4	—	—
			Av.	+0·1	− 1·8	−10·1	0·3	0	—
0·4	25°	30					0	0·7	—
,,	,,	,,		+0·3	− 2·4	− 8·3	0	0·4	—
,,	,,	,,		+0·7	− 2·2	− 7·9	0·4	—	—
,,	,,	,,		0	—	—	0·4	—	—
,,	,,	,,		0	—	—	0	—	—
,,	,,	,,		0	—	—	0	—	—
,,	,,	,,		0	—	—			
			Av.	+0·2	− 2·3	− 8·1	0·1	0·5	—
0·8	21°	30		−8·8	—	—	—	—	—
,,	,,	,,		−6·7	−20·3	−30·8	0·9	0·9	—
,,	,,	,,		−8·8	—	—	—	—	—
,,	,,	,,		−8·8	—	—	—	—	—
,,	,,	,,		−9·7	−20·5	—	2·6	0·9	—
			Av.	−8·6	−20·4	−30·8	1·7	0·9	—

(2) Under the conditions necessary for the complete oxidation of 1 mg. glucose, fructose is also oxidised to a significant extent, which may amount to 9 % in 20 minutes at 21°. This disadvantage is not overcome by using other proportions of sodium hydroxide.

(3) With sodium carbonate in suitable amounts (0·2 cc. or 0·4 cc. 5 % Na_2CO_3), oxidation of 1 mg. glucose is complete in 30 minutes at 21°; 2 mg. glucose are oxidised to the extent of 98 %, but with larger amounts the results are low and irregular. Fructose is oxidised only to a very small extent, the values shown (0–2 %) lying close to the limits of titration errors by this method.

(4) With amounts of 5 % sodium carbonate less than 0·2 cc. or more than 0·4 cc. the oxidation of glucose is incomplete.

From other tests it was found that variations in the temperature between 21° and 25°, or in the time between 20 and 45 minutes did not affect the results. The use of sodium carbonate therefore offers a greater latitude as regards amount of alkali, temperature etc. than is permissible with sodium hydroxide.

For routine estimations the conditions adopted were, for amounts of sugar equivalent to 1–1·5 mg. glucose, 3 cc. 0·02 *N* iodine; 0·2 cc. 5 % Na_2CO_3; 21° and 30 minutes. These correspond closely with the conditions recommended by Bougault for larger quantities of sugar.

Table IV gives the average results obtained with various sugars under the above conditions. For the aldoses the results are stated as percentages of the respective compounds calculated from the iodine reduced, according to the equation. For the ketoses and sucrose the results are given in terms of the equivalent quantity of glucose. The results for galactose were persistently low and were not improved by increasing the time or by adding the alkali over a period of 6 minutes[1]. In this table are also included values obtained for barium glucosemonophosphate, barium fructosemonophosphate (Neuberg) and barium fructosediphosphate, those for the fructose derivatives being calculated as percentage glucose. In spite of the low value for the glucosemonophosphate there is good reason for believing that the specimen was free from fructosemonophosphate (Robison and King, unpublished work).

It should perhaps be emphasised that great caution is necessary in interpreting the results obtained by this method with crude biochemical products since many compounds other than aldoses may react with iodine under these conditions.

Table IV.

Sugar	1 mg.	2 mg.	3 mg.	4 mg.
Glucose	100·6	97·8	78·1	—
Galactose	97·0	96·5	85·6	—
Maltose	102·8	100·9	—	78·4
Lactose	102·3	98·3	—	75·5
Fructose	0·8	1·6	1·8	—
Sucrose	2·0	0·8	1·7	—
Barium glucosemonophosphate	—	90·2	—	—
Barium fructosemonophosphate	—	—	—	(15 mg.) 3·0
Barium fructosediphosphate ...	—	—	—	0–2·0

Some experiments were carried out to determine to what extent the Lobry de Bruyn and van Ekenstein transformation may account for the high values obtained for fructose using sodium hydroxide. In order to increase the effect, the ratio of sodium hydroxide to iodine was raised to 10 : 3 but the concentration of alkali was only 0·03 *N*. The results are set out in Table V.

[1] *Note (added 7th June* 1929). Satisfactory results have, however, since been obtained with galactose, prepared by the hydrolysis of a highly purified specimen of α-methyl-*d*-galactoside, for which we have to thank Mr J. A. Pryde.

Column A. Reducing power of fructose by the usual carbonate method (in this case, 0·4 cc. 5 % Na_2CO_3 was used) 30 minutes at the specified temperatures.

Column B. Reducing power using 2 cc. 0·1 *N* NaOH, 30 minutes at the specified temperature.

Column C. Fructose solution allowed to remain for 30 minutes at the specified temperature in presence of 2 cc. 0·1 *N* NaOH, then neutralised and reducing power estimated by the carbonate method (30 minutes, 21°).

Column D. Fructose solution allowed to remain for 30 minutes at the specified temperature in presence of 2 cc. 0·1 *N* NaOH. Iodine then added and solution left for further 30 minutes at the specified temperature.

Table V.

Amount of fructose mg.	Temp.	*A*	*B*	*C*	*D*
1	21°	0·4	13·1	5·3	24·9
5	21°	0·6	16·8	4·4	16·9
1	25°	0·4	17·8	11·4	25·8
5	25°	0·9	17·2	6·2	22·2
1	38°	4·0	68·0	22·7	84·1
5	38°	2·5	30·4	14·7	39·4

Comparison of columns *A* and *C*, as also of columns *B* and *D*, shows that some change is produced by the action of the alkali alone, but this could at most account for only a fraction (30–40 %) of the total oxidation effected by the combined action of the iodine and alkali, shown in column *B*, unless the nett rate of the transformation of fructose into glucose is greatly increased in presence of the iodine owing to the rapid removal of the aldose by oxidation.

Summary.

The conditions for the estimation of very small amounts of aldoses by the iodimetric method have been investigated. Satisfactory results are obtained using 3–4 times the theoretical quantity of iodine with sodium carbonate as the alkali, and allowing the oxidation to proceed during 30 minutes at 21°. Under these conditions the oxidation of glucose is complete while fructose and sucrose are only oxidised to a very small extent.

Values obtained for other sugars and for hexosemono- and hexosediphosphates are also given.

The extent to which fructose is oxidised by iodine in presence of excess of sodium hydroxide is much greater than can be explained by the Lobry de Bruyn and van Ekenstein transformation unless it is assumed that the change from ketose to aldose is largely increased owing to the rapid and continuous removal of the latter by oxidation.

We are greatly indebted to Mr J. L. Baker and Mr H. F. E. Hulton for kindly supplying us with several specimens of pure sugars.

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LX. THE CHEMICAL CONSTITUTION OF THE GUMS.

PART I. THE NATURE OF GUM ARABIC AND THE BIOCHEMICAL CLASSIFICATION OF THE GUMS.

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Preamble.

The latest work which has been carried out on gums is not of very recent date, and advances in carbohydrate chemistry which have been made since that time make it desirable that this work should be repeated. The physical difficulties of working with these substances are considerable, and the situation is aggravated by the fact that the natural gums are frequently composed of mixtures of several individual types. Separation of such mixtures is very difficult, and it is probable that physical methods based on their colloidal properties will have to be elaborated.

Early investigators have shown that the hydrogen and oxygen in gums exist in approximately the same proportions as in water, and accordingly the gums were considered to be true carbohydrates. Later, by the action of acids, various sugars were obtained from them by hydrolysis, and the gums were thereupon classified as polysaccharides. Still later, it was demonstrated that they are not of a true polysaccharide nature, since they consist of numbers of sugar molecules united to a central nucleus of a sugar acid nature. The attachment seems to be of an ethereal or glucosidic type, and not an ester type, since the gums retain acidic properties, and are capable of forming salts. In point of fact, they are usually found as salts of calcium, magnesium, or potassium.

It is to be emphasised that the classification of a substance as a gum is as yet mainly an arbitrary one, not being based on any constitutional knowledge, but decided by its origin and physical behaviour, together with the fact that it is of a carbohydrate nature. When the chemical composition of these substances has been determined, it may be possible to suggest a more rational grouping, and also to speculate with some degree of probability as to their rôle in nature.

The first member of this group to be studied was gum arabic, since from the work of previous investigators this appears to be a single substance.

THE COMPOSITION OF GUM ARABIC.

Introduction.

The early investigators of the composition of gum arabic seem chiefly to have been concerned in attempting to assign to it an empirical formula, and several workers in the first half of the last century gave analyses closely approximating to $(C_6H_{10}O_5)_n$. Neubauer [1854, 1857, 1863] seems to have been the first to record the fact that gum arabic is an acid. He prepared pure salts of this acid, and subjected them to analysis, from the results of which he deduced various empirical formulae for the salts.

O'Sullivan [1884] described some very careful and detailed investigations into the nature of this substance. His methods were, briefly, hydrolysis with acid, preparation of the barium salt of the hydrolysis product, fractional precipitation with alcohol, if necessary, and ultimate analysis of the salt obtained. By these means he concluded that this gum had an empirical formula of $C_{89}H_{142}O_{74}$ and consisted of molecules of four, or possibly five, hexose sugars, termed by him α-, β-, γ-, and δ-arabinoses, linked to a nucleus of a comparatively stable nature to which he assigned the empirical formula of $C_{23}H_{38}O_{22}$, and the name "arabinosic acid." In a later paper, O'Sullivan [1889] recognised the presence of only two sugars in this gum, a pentose, arabinose, and a hexose, galactose, and suggested a more extended formula, which will be quoted later.

Experimental.

A good commercial sample of gum arabic is a light transparent substance, slightly yellowish in colour, in droplets with a vitreous fracture. The ash content varies from 3 to 4 % or more, and there is some moisture present. This gum is found naturally as the salts of calcium, potassium, and magnesium. Pure acid gum arabic is prepared from the natural product according to the method employed by Neubauer [1854].

The gum is dissolved in warm water to give a fairly concentrated solution, which is then made acid by the addition of hydrochloric acid. Insoluble particles are allowed to settle, and the clear liquid is decanted off. The acid gum arabic is then precipitated by the addition of alcohol. Precipitation takes place when the alcohol content is as low as 50 %, but as a general rule this should be raised to 60 % so that precipitation may be complete. The acid gum arabic appears as a thick and curdy precipitate. This is filtered off, re-dissolved in warm water and acidified by the addition of a little hydrochloric acid just previous to precipitation with alcohol as before. This process is repeated at least three times. The final product is dried in absolute alcohol, and in a desiccator over phosphorus pentoxide. By this means an extremely pure product is obtained, the ash content of which is very low.

It was considered likely that the nucleus-acid of gum arabic, termed by O'Sullivan first "arabinosic acid" and later "arabic acid," might be of the

nature of a "uronic acid." Accordingly the yield of carbon dioxide on boiling with 12 % hydrochloric acid was determined in the same way as for pectin products [Nanji, Paton and Ling, 1925].

Acid gum arabic. Sample I.	Ash	0·26 %
Yield of CO_2 on an ash-free basis	(i)	3·11 %
	(ii)	3·17 %
	Mean	3·14 %
Corresponding to uronic acid anhydride		12·56 %

Since this gum has been shown by many workers to contain pentose, the furfuraldehyde yield was determined by distillation and precipitation as phloroglucide. The precipitated phloroglucide was in each case extracted with absolute alcohol, since the presence of methylpentose in this gum has been suggested. Although by this extraction a considerable amount of substance was removed, concurrent figures were never obtained. Further the alcoholic extract was never of a reddish colour, as it is stated to be in the presence of the phloroglucide of methylfurfuraldehyde, but always a deep green. The substance removed is probably the phloroglucide of ω-hydroxymethylfurfuraldehyde, obtained in small quantities from the hexosan units.

Acid gum arabic. Sample I.

Yield of furfuraldehyde on ash-free basis	(i)	16·45 %
	(ii)	16·01 %
	(iii)	16·25 %
	Mean	16·24 %

Now it is known that the uronic acid anhydride units yield 16·66 % of their weight of furfuraldehyde, and consequently the 12·56 % of uronic acid anhydride which is present in this sample of acid gum arabic accounts for 2·09 % furfuraldehyde; the balance of furfuraldehyde, 14·15 %, being due to pentose.

Many workers have stated that the sugars to be obtained by the hydrolysis of this gum are arabinose and galactose. This statement was confirmed. It is not necessary to describe in detail the procedure, but it is sufficient to say that the characteristic osazones of arabinose and galactose were obtained from the hydrolysis liquid after removal of the acid. Arabinose diphenylhydrazone was prepared by treatment of the syrup with excess of diphenylhydrazine in alcoholic solution according to the method of Neuberg [1900]. The recrystallised hydrazone had a melting point of 202° (Neuberg: 204°). Mucic acid was also obtained by treatment with nitric acid. No trace of xylose could be detected by use of Bertrand's cadmium xylonobromide reaction; neither was any hexose other than galactose traced. It seems, then, that the only sugars formed on hydrolysis are arabinose and galactose.

It is clear then that the furfuraldehyde in the molecule due to pentose may be calculated as arabinose, indicating that there is present 26·31 % anhydro-arabinose ($C_5H_8O_4$), yielding on hydrolysis 29·89 % of arabinose.

No satisfactory quantitative method has ever been described for the estimation of galactose, the oxidation by nitric acid to mucic acid giving invariably a low result, the production of mucic acid being accompanied also by the formation of oxalic acid.

It is only possible, therefore, to arrive at figures for galactose by difference.

Acid gum arabic. Sample I.

Uronic acid anhydride	12·56 %
Anhydro-arabinose	26·31 %
Anhydro-galactose (by difference)	61·13 %

yielding on hydrolysis, arabinose 29·89 % and galactose 67·92 %.

Much of the work of O'Sullivan is based on the estimation of the barium content of the barium salt, and although this undoubtedly gives a fair indication of the carboxyl groups present, it does not seem that it can be taken as an absolute figure, unlike the determination of carbon dioxide yield in this work. The method followed by him in the preparation of the barium salt of acid gum arabic was exactly to neutralise a solution of the acid with baryta water, and then to precipitate with alcohol, and dry. Since the acid is a very weak one, it is difficult to determine the precise neutral point. The alcohol precipitate is of a bulky nature, and would undoubtedly carry down with it any excess baryta which might be present, and so vitiate the results. Alternatively, if the end-point were not quite reached, the results might be low owing to the presence of free acid gum arabic.

The barium salt of acid gum arabic was prepared by careful addition of baryta as described by O'Sullivan. The amount of baryta to be added was ascertained on a duplicate, using phenolphthalein as indicator. After filtration, the salt was precipitated with alcohol, washed, and dried. A barium estimation was carried out on this preparation, gravimetrically as sulphate.

Barium content of salt of acid gum arabic.

Preparation (i)	5·07 % Ba
,, (ii)	5·27 % Ba

Now, the yield of carbon dioxide from the preparation indicates the presence of carboxyl groups which would only be capable of uniting with 4·89 % barium. There is the possibility, of course, that there might be present in the nucleus of this gum some simple organic acid, not of a uronic type, not yielding carbon dioxide under the conditions of that estimation. This, however, is very unlikely, since no such acid has been discovered in the hydrolysis liquid, and further, the difference between the theoretical barium content and that actually found is only small, and is to be anticipated from the method of preparation. It seems, therefore, that this particular method, the preparation of the barium salt and the subsequent estimation of its barium content, is not reliable as giving an indication of the amount of carboxyl groups present. Very many of O'Sullivan's conclusions are based upon the results

of this determination, and consequently they cannot be accepted as absolute figures, though, as seen above, it is likely that they are fair approximations.

O'Sullivan in his earlier paper on this subject considered all the sugars split off to be of a hexose nature, but after the discovery of the pentose nature of arabinose he assigned to acid gum arabic the following formula:

$$2(C_{10}H_{16}O_8).4(C_{12}H_{20}O_{10}).(C_{23}H_{30}O_{18}).$$

He described it as "di-arabinan-tetra-galactan-arabic acid," the term "arabic acid" being employed for the nucleus-acid in place of the term "arabinosic acid" employed by him previously. It is necessary to note that the "arabic acid" of other workers corresponds with that which, to prevent confusion, is described in this paper as "acid gum arabic." In the formula above "arabinan" and "galactan" respectively stand for two molecules of arabinose and galactose less two molecules of water.

By calculation, such a molecule consists of

anhydro-arabinose $(C_5H_8O_4)_n$	21·84 %
anhydro-galactose $(C_6H_{10}O_5)_m$	53·60 %
anhydro-"arabic acid"	24·56 %

yielding on hydrolysis, arabinose 24·82 % and galactose 59·57 %.

It will be seen that these figures differ very considerably from those obtained experimentally in this paper, the difference between the anhydro-"arabic acid" of O'Sullivan's formula and the uronic acid anhydride of the preparation being particularly marked. On the other hand, it must not be assumed that all samples of gum arabic are identical in composition. Such is quite definitely not the case, though they appear to be similarly constituted. This means, however, that it is not possible to assign to this substance any definite empirical formula, but only to indicate the constituents and the probable method of linkage.

Sample II of gum arabic, treated as before to obtain the acid gum arabic, gave on analysis the following figures:

Acid gum arabic. Sample II. Ash 0·24 %.

Furfuraldehyde yield on ash-free basis	(i)	14·00 %
	(ii)	13·86 %
	(iii)	13·98 %
	(iv)	13·89 %
	Mean	13·93 %
Yield of carbon dioxide on ash-free basis	(i)	4·36 %
	(ii)	4·42 %
	Mean	4·39 %

By calculation

uronic acid anhydride	17·56 %
furfuraldehyde due to uronic acid anhydride	2·91 %
balance of furfuraldehyde due to pentose	11·02 %

The figures for Sample I, and those obtained by calculation from O'Sullivan's formula, are quoted for comparison.

	Sample I	Sample II	O'Sullivan
Uronic acid anhydride	12·56	17·56	(anhydro-"arabic acid" 24·56)
Anhydro-arabinose	26·31	20·52	21·84
Anhydro-galactose	61·13	61·92	53·60
Yielding on hydrolysis:			
Arabinose	29·89	23·32	24·82
Galactose	67·92	68·80	59·57

By hydrolysis with acids O'Sullivan was able to arrive at conclusions stated to justify his formula. He was, however, largely concerned with the identification of the sugars liberated, and the barium content of the salts of the acid residues isolated. He appears to have chosen concentrations of sulphuric acid for hydrolysis in a rather haphazard way. In the work described here, it was thought desirable to adhere throughout to one concentration, namely, 3 %. The hydrolysis was carried out by O'Sullivan in the following manner. To the acid gum arabic in solution was added the necessary amount of sulphuric acid, and the whole boiled. At the conclusion of the period of hydrolysis the solution was cooled, and neutralised with a hot solution of baryta. The precipitated sulphate was filtered off, and the filtrate evaporated and poured into excess of alcohol. The precipitate was repeatedly dissolved in water and reprecipitated by alcohol.

This method was followed for a time, with the difference that carbon dioxide was passed through after the addition of the baryta to remove any excess. It was found, however, that by this method it was not possible entirely to free the hydrolysis product from the sugars liberated at the same time, and as a result sticky or slimy precipitates were obtained. Also the ash content of those that were obtained in a reasonably friable condition was unduly high.

Accordingly the method was modified. At the close of the period of hydrolysis, while still very hot, the sulphuric acid employed is nearly neutralised by the addition of solid barium carbonate in a very fine condition. After standing for a few moments, the precipitated sulphate is filtered off. Immediately, while the filtrate is still hot, boiling alcohol is poured in until the concentration of alcohol reaches 60 %. The precipitate which forms settles quickly, and the supernatant liquid is poured off before it has cooled. Since hot alcohol of a concentration of 60 % does not precipitate either galactose or arabinose, the end-product obtained by this means is free from these substances. The precipitate is dissolved in water, filtered, heated nearly to boiling and reprecipitated by hot alcohol as before. This is repeated several times. The final product obtained is not sticky, and on being brought into contact with absolute alcohol it becomes white and friable. It will be noticed that by this method the free acid hydrolysis product is obtained in place of the barium salt as by the method of O'Sullivan.

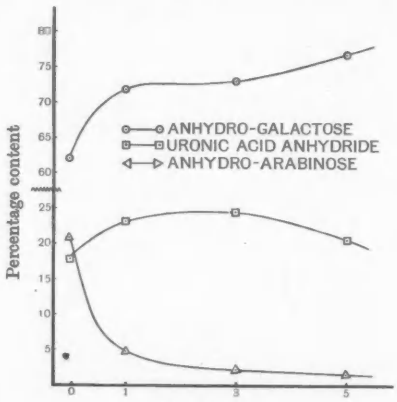
Hydrolyses were carried out for periods of 1, 3, 5, 6, 7 and 10 hours with

3 % sulphuric acid. It is inadvisable to commence with less than 200 g. in each case for the yields are small. Three times the weight of sulphuric acid was employed. No substance remained precipitable by hot alcohol after either 10 or 7 hours' hydrolysis. Only traces precipitable by alcohol were found after 6 hours, the quantity being quite insufficient for analysis.

The following were the analytical figures obtained, in each case being the mean of two or more closely agreeing determinations. All hydrolyses were carried out upon Sample II of gum arabic.

	Acid gum arabic Sample II	Product after 1 hour's hydrolysis	Product after 3 hours' hydrolysis	Product after 5 hours' hydrolysis
	%	%	%	%
Ash	0·24	2·87	3·32	3·91
Furfuraldehyde yield (ash-free) ...	13·93	6·48	5·22	4·28
CO_2 yield (ash-free)	4·39	5·79	6·13	5·25
Uronic acid anhydride	17·56	23·16	24·52	21·00
Furfuraldehdye due to uronic acid	2·91	3·86	4·08	3·49
Balance of furfuraldehyde	11·02	2·62	1·14	0·79
Anhydro-arabinose	20·52	4·93	2·12	1·48
Anhydro-galactose	61·92	71·91	73·36	77·52
Yielding arabinose	23·32	5·60	2·41	1·68
Yielding galactose	68·80	79·91	81·53	86·14

From a consideration of these figures it is possible to obtain some insight into the composition of this gum. The results are even clearer when plotted as a graph. It will be seen from the graph that the arabinose is rapidly removed from the molecule during the first hour of hydrolysis, and the amounts of uronic acid anhydride and of anhydro-galactose rise sharply, proportionately,



Hydrolysis of gum arabic with 3 % sulphuric acid

and approximately evenly. During the second and third hours this condition is maintained at a much slower rate, and by this time practically all the arabinose has been removed. After the third hour it is seen that the uronic acid anhydride tends to diminish a little, and the anhydrogalactose to rise.

It is quite evident, therefore, that the linkage of the arabinose units is different in nature from that between the other units, since it is very much weaker, being ruptured by brief treatment with acid. Knowing that gum arabic is not a reducing body it is probable that the linkage between the arabinose units and the nucleus-acid is an ethereal or glucosidic linkage.

It would seem that the nucleus-acid, the "arabic acid" of O'Sullivan, is composed of galactose and the uronic acid, probably in the proportion of two or three molecules of the former to one of the latter, or some multiple of these figures. This is a little surprising, but even more unusual is the fact that the galactose does not appear to be split off except when the nucleus-acid is completely ruptured. That this must take place continuously during the hydrolysis is evidenced by the fact that galactose in some quantity can be obtained from the hydrolysis liquid after less than 3 hours' hydrolysis. Nevertheless the uronic groups are apparently removed somewhat more easily than the galactose units. The nature of the uronic acid has not been definitely established. It is, however, in all probability, galacturonic acid, since glycuronic acid, although excreted in certain abnormal conditions of animal metabolism, has never been unquestionably proved to be a constituent of plant materials, save possibly in the hemicellulose A isolated by O'Dwyer [1926] from beechwood.

Discussion.

Work which has been carried out on other gums has shown that they also are constructed in much the same general way as gum arabic, namely by linkage of hexose or pentose sugars with uronic acid units. It is possible, therefore, tentatively to suggest a position for the gums in the general field of plant biochemistry. Candlin and Schryver [1928] have suggested the use of the word "polyuronide" to describe a substance which consists of sugars linked to a uronic acid. It is evident, then, that the gums may be described as "polyuronides" if this term be accepted.

Table I. *Uronic acid anhydride content of some plant products.*

Substance	Source	Uronic content %	Sugars present	Authority
Pectic acid	Lemons	70·56	Galactose, arabinose	Nanji, Paton and Ling [1925]
Hemicellulose B	Beechwood	64·0	Galactose, arabinose	O'Dwyer [1926]
Mild oxidation products of pectin	Lemon pectin	60–55	Galactose, arabinose	Norman (unpublished)
Tragacanthin	Gum tragacanth	50·8	Arabinose	Norman (unpublished)
Hemicellulose by alkali treatment	Citrus pectin	37·3	Presumably galactose, arabinose	Candlin and Schryver [1928]
Hemicellulose B	Oats straw	31·8	? ?	Norman (unpublished)
Bassorin	Gum tragacanth	30·0	Arabinose, galactose, xylose?	Norman (unpublished)
Hemicellulose by alkali treatment	Onion pectin	21·5	Presumably galactose, arabinose	Candlin and Schryver [1928]
Acid gum arabic	(Sample II)	17·56	Galactose, arabinose	Norman
Hemicellulose B	Beechwood	18·8	? ?	Candlin and Schryver [1928]
Hemicellulose A	Turnip	15·4	? ?	Candlin and Schryver [1928]
Acid gum arabic	(Sample I)	12·56	Galactose, arabinose	Norman
Hemicelluose A	Beechwood	11·5	? ?	Candlin and Schryver [1928]
Hemicellulose A	Beechwood	11·0	Xylose (galactose?)	O'Dwyer [1926]
Hemicellulose A	Oats straw	10·8	Arabinose ?	Norman (unpublished)
Hemicellulose B	Turnip	10·7	? ?	Candlin and Schryver [1928]

Several recent workers have shown that natural hemicelluloses consist of hexose and pentose sugars linked together and usually in combination with a uronic acid. There does not seem to be, therefore, any essential difference in general structure between the gums and the hemicelluloses, which is rather supported by the fact that they are not dissimilar in physical properties and solubilities. It is rapidly becoming clear that the uronic acids are much more widely distributed in vegetable materials than was previously supposed (see Table I[1]).

Candlin and Schryver [1928] have recently demonstrated that pectin may be decarboxylated by treatment with alkali, the degree of decarboxylation depending upon the concentration and temperature of the alkali, with the resulting production of substances of a hemicellulose type, resembling in all respects the hemicelluloses isolated directly from wood. It would seem, then, not unlikely that in nature both hemicelluloses and gums may be produced by partial decarboxylation of pectin. This change may be brought about *in vitro* not only by alkali treatment but also by mild oxidation, on which point details of some work will shortly be published. Such a process as mild oxidation more nearly approaches, of course, the changes which may possibly take place in the living plant.

Both von Fellenberg [1918] and Ehrlich [1927] have suggested the possibility that the pectic substances may undergo transformation to lignin, and O'Dwyer [1928] states that it is not unlikely that hemicellulose may be a transition product. She showed [1926] that unlignified tissues contain a much larger amount of pectin than of hemicelluloses, while in lignified tissues the opposite holds, in fact only the merest traces of pectin are usually present. Candlin and Schryver [1928] repeated the suggestion that lignin may be derived from pectin, but their experimental work, which resulted in the artificial production of a hemicellulose from pectin, did not lend support to this theory. It must be admitted that it makes an attractive theory to couple the two substances, pectin and lignin, since the observation that pectin decreases on lignification seems to hold good generally, but nevertheless on purely chemical and constitutional grounds it is not at present easy to see how the transformation may take place. Further, the actual amount of lignin present in lignified tissue is very considerably in excess of the amount of pectin which was present in the same tissue before lignification. On quantitative grounds also, there is considerable support for the view that pectin is converted into hemicellulose as the tissue becomes older. Though this process may be coincident with the development of lignification, it is not possible from present evidence to assume any interrelation.

On purely chemical grounds it is possible to account for the formation of substances of the nature of pectin, hemicelluloses and gums, by the protracted

[1] It is probable, if not certain, that several of the products mentioned in this table, notably the hemicelluloses, are mixtures, and if suitable means of separating them can be elaborated even more significant uronic figures may be obtained.

mild oxidation of linked hexose units. For example, when *d*-glucose is linked in some form as a polysaccharide through its carbonyl groups, mild oxidation will have the effect of converting the terminal carbinol groups to carboxyl, and so, in a simple hypothetical case, forming a conjugated *d*-glycuronic acid, which on complete decarboxylation yields a xylan: similarly *d*-galactose might be supposed to give rise to an araban. If oxidation is only partial, that is to say, if some units of the hexosan are incompletely oxidised, then other complex substances containing hexose, uronic and pentose groups would be formed, akin in all respects to pectin, hemicelluloses and gums. It is undoubtedly a fact that it is the derivatives of galactose, possibly formed in this way, which are met with most frequently in the substances classed as hemicelluloses and gums, and further, each of the units in pectin is derivable from galactose. Xylans and gluco-xylans are relatively less common and widespread than the galacto-arabans. It is curious that galactose thus appears to occupy a place rather more important in this transition to pentosans than does glucose, while it is glucose which is the most widespread unit of hexosans, *e.g.* in starch and cellulose. The change from glucose to galactose is one which must take place readily in plants, and also in animals, as evidenced by the production of lactose on a diet free from galactose. The explanation of this prominence of galactose and its derivatives in plant constituents is not obvious, unless it be that hexose in the form of galactose is less readily respired than as glucose.

It would seem that the first apparent stage in the oxidation of galactans is the production of pectin, and having regard to the flexibility of the process of mild oxidation, it is perhaps remarkable that a substance of such definite character as pectin should appear. The homogeneity of pectin preparations from many sources appears to have been established by the work of Clayson, Norris and Schryver [1921], and of Norris and Schryver [1925]. Recently, however, Ehrlich and Schubert [1926] have claimed that pectin from flax contains xylose, in addition to galactose, galacturonic acid, and arabinose. Henderson [1928], on rather insufficient evidence, has claimed, on the contrary, that pectin from flax contains only galactose and galacturonic acid. In view of this conflict of opinions this form of pectin was re-investigated by Norris [1929], who finds no reason for departing from the original six ring formula put forward by Nanji, Paton and Ling [1925].

The formation of pectin in the plant appears to take place when metabolism is at its highest peak, that is when growth is most rapid. As growth slows down and maturity is reached, so does the production of pectin decrease, and that present is slowly converted into other substances, probably hemicelluloses and gums. It seems likely, if not certain, that the hemicelluloses and gums are not substances definite in composition but are built up on a general plan from the intermediate oxidation products of hexosans. If the oxidation is prolonged, it may be that complete decarboxylation follows, so that true pentosans would be formed. It must be admitted, however, that

it has yet to be demonstrated that such substances as true pentosans have any existence in fact. Examinations of preparations hitherto regarded as being true pentosans have shown that a small quantity of uronic acid is present, and this, it is claimed, is the clue to their formation.

SUMMARY.

1. It is shown that the acid group in gum arabic is of the uronic type.
2. A method of isolation of the acid hydrolysis products is given.
3. The statement that the only sugars present in gum arabic are galactose and arabinose is confirmed; analytical figures are given for these substances, both in the untreated gum and in the hydrolysis products.
4. It is clear that gum arabic is not a substance of definite empirical formula. It is possible, however, to indicate its general composition—a nucleus-acid consisting of galactose and a uronic acid, probably galacturonic acid, to which is linked arabinose by glucosidic linkages. The arabinose is in consequence more easily split off than the other components.
5. There seems to be no essential difference in structure between gums and hemicelluloses, both consisting of hexose and pentose sugars linked to uronic acids.
6. It is suggested that it is by the protracted mild oxidation of linked hexose, and particularly galactose units, that pectin and the hemicelluloses and gums are formed.

In conclusion it is a pleasure to express my thanks to Prof. A. R. Ling and Dr F. W. Norris for their assistance and advice.

Addendum (June 8th, 1929). While the above communication was in the press, Butler and Cretcher (*J. Amer. Chem. Soc.* 1929, **51**, 1519) have published some work on the constitution of this gum. They claim to have isolated an aldobionic acid, galactose-glucuronic acid, corresponding to the nucleus-acid of O'Sullivan. To this are attached galactose, arabinose, and rhamnose. They do not appear to recognise the fact that gum arabic is not a specific substance. Further, it is not possible to agree that all the phloroglucide soluble in alcohol must be derived from methylpentose, for it is well known that under the conditions obtaining in this estimation, hexosan units yield a small but inconstant amount of ω-hydroxymethylfurfuraldehyde, the phloroglucide of which is soluble in alcohol. Accordingly the figure quoted by them for rhamnose is probably much too high. On the other hand it is not unlikely that that for galactose is too low, since the mucic acid method is not strictly quantitative.

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LXI. OBSERVATIONS ON THE CARBOHYDRATE METABOLISM OF TUMOURS.

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The studies of Warburg and his collaborators on the carbohydrate metabolism of surviving tumour tissues [1926] have dealt mainly with two transplantable strains of rat tumours, Jensen's rat sarcoma and Flexner's rat carcinoma, and the Rous chicken sarcoma.

On the basis of metabolism values found for a large number of these tumours, together with those obtained for a more limited series of human neoplasms, Warburg has suggested several generalisations, showing characteristic relationships between the magnitudes of the respiration and the aerobic and anaerobic glycolysis. The constant result which emerged was the abnormally high value of the anaerobic glycolysis as compared with the respiration.

Assuming that the oxygen utilised was functioning at its maximum efficiency in causing the removal or non-formation of lactic acid under aerobic conditions, the respiration was found inadequate to check the glycolysis completely, a relatively large excess fermentation remaining.

Representing this excess fermentation by U, the respiration by Q_{O_2}, and the anaerobic glycolysis by $Q_M^{N_2}$

$$U = Q_M^{N_2} - 2\,(Q_{O_2}).$$

Positive values for U were invariably found for the tumours quoted.

Aerobic glycolysis is not a specific feature of tumour tissue. Warburg [1929] has recently summarised examples of normal tissues possessing aerobic glycolysis, and Crabtree [1928] showed it to be a property of certain pathological overgrowths associated with intracellular viruses.

During the last year, as material has become available, the carbohydrate metabolism of several strains of transplantable mouse tumours, propagated in this laboratory, has been measured, and the results are collected in Table I. Although Warburg no longer adheres to the classification of tissues on the basis of a positive or negative value of U, throughout this communication the value will be introduced as affording an arbitrary standard of comparison, placing the present measurements in line with those previously recorded.

The manometric technique elaborated by Warburg has been followed throughout.

Table I. *Carbohydrate metabolism of mouse tumours.*

Tumour	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Meyerhof quotient	U
Crocker sarcoma	−10·6	+14·8	+30·4	1·5	+ 9·2
,,	−14·6	+15·6	+25·2	0·6	− 4·0
,,	−30·9	+20·7	+31·7	0·4	−30·1
,,	−13·9	+15·0	+26·5	0·8	− 1·3
,,	− 8·2	+16·3	+26·5	1·2	+10·1
,,	−16·5	+16·2	+30·5	0·9	− 2·5
,,	− 7·6	+13·2	+30·6	2·3	+15·4
,,	−15·5	+17·3	+24·8	0·5	− 6·2
,,	−24·4	+19·4	+24·9	0·2	−23·9
,,	−11·9	+16·7	+22·1	0·5	− 1·7
,,	− 6·5	+12·6	+23·2	1·6	+10·2
,,	−20·7	+19·7	+31·3	0·6	−10·1
,,	−21·4	+20·9	+33·3	0·6	− 9·5
Tar carcinoma (Sp) 2146	−19·3	+19·6	+23·5	0·2	−15·1
,,	−21·3	+18·4	+24·5	0·3	−18·1
,,	−15·2	+14·3	+24·7	0·7	− 5·7
,,	−31·0	+18·6	+25·7	0·2	−36·3
,,	−19·5	+11·6	+24·0	0·5	−15·0
,,	−13·3	+ 8·5	+24·0	0·9	− 2·6
37 sarcoma	−10·9	+12·6	+23·5	1·0	+ 1·7
,,	− 9·8	+15·3	+29·4	1·5	+ 9·8
,,	−12·2	+ 6·5	+26·6	1·6	+ 2·2
,,	−12·1	+ 6·9	+27·6	1·6	+ 3·4
,,	−22·3	+14·4	+32·4	0·8	−12·2
,,	−24·3	+14·8	+33·4	0·7	−15·2
Tar sarcoma Bonné	−15·9	+ 9·1	+20·7	0·7	−11·1
,,	−13·9	+ 8·6	+20·8	1·0	− 7·0
,,	− 9·9	+12·3	+24·9	1·3	+ 5·1
Tar carcinoma 173	−12·0	+15·2	+30·7	1·3	+ 6·7
,,	−13·0	+16·3	+24·1	0·6	− 1·9
,,	−20·1	+15·4	+31·9	0·8	− 8·3
,,	−16·6	+20·7	+28·9	0·5	− 4·3
,,	−17·0	+19·5	+28·9	0·5	− 5·1
,,	−16·7	+17·0	+36·0	1·1	+ 2·6
,,	−14·4	+18·9	+37·0	1·2	+ 8·2
,,	−14·5	+10·2	+21·5	0·8	− 7·5
,,	− 8·2	+ 6·7	+21·5	1·8	+ 5·1
,,	−10·0	+ 8·2	+26·5	1·8	+ 6·5
,,	−18·3	+11·6	+27·5	0·8	− 9·1
Sarcoma 2529	−14·8	+14·9	+32·6	1·2	+ 3·0
,,	− 5·6	+12·8	+30·6	3·5	+19·4
,,	−16·5	+17·8	+36·3	1·1	+ 3·3
,,	−19·6	+22·1	+36·9	0·8	− 2·3
,,	−16·0	+13·5	+28·3	0·9	− 3·7
,,	−11·6	+12·7	+28·3	1·3	+ 5·1
,,	−10·9	+14·8	+29·7	1·4	+ 7·9
,,	−12·9	+15·1	+28·7	1·1	+ 2·9
Glycogen-carcinoma 113	−15·5	+ 6·8	+12·6	0·4	−18·4
,,	−12·5	+ 5·0	+12·7	0·6	−12·3
,,	− 8·3	+ 3·4	+14·4	1·3	− 2·2
,,	− 7·6	+ 2·8	+14·1	1·5	− 1·1
,,	−11·4	+ 4·3	+16·1	1·0	− 6·7
Melanotic sarcoma	− 5·6	+ 7·2	+23·7	2·9	+12·5
,,	− 8·8	+ 4·3	+15·3	1·3	− 2·3
,,	− 7·7	+ 4·4	+15·1	1·4	− 0·3
,,	−11·7	+ 7·0	+ 8·9	0·2	−14·5

The composition of the saline medium used, unless otherwise stated, was:

Salt	Moles per litre
NaCl	0·121
KCl	0·0025
$CaCl_2$	0·0018
$NaHCO_3$	0·025

Glucose was added in a concentration of 0·2 %, and the appropriate gas phase was 5 % CO_2 in O_2 or in N_2.

The method of expressing results is that used by Warburg and is explained in an earlier communication of the present author [1928].

Though every precaution was taken to ensure efficient metabolism, by using thin sections which permit perfect diffusion of metabolites, and by removal of all traces of necrosis visible to the naked eye, the results obtained were widely divergent in character.

The great variability in the absolute and relative magnitude of the metabolism values is not merely between the various strains investigated, but is apparent among different tumours of the same strain.

About one-third show values comparable to those found for Jensen's rat sarcoma and Flexner's rat carcinoma (see Table VI). The majority deviate from these standards, chiefly by exhibiting a higher respiration, both in its absolute value and also in its relation to the aerobic and anaerobic glycolysis. The relationship is emphasised by the negative value of U in numerous cases.

Many factors could conceivably operate in contributing to these abnormal findings: the generally more active metabolism of the mouse compared with larger animals, the variations in the environment at the site of growth, the possible fluctuations in the respiratory quotient, or the effectiveness of the blood supply. Also, in some tumour strains phases of rapid growth alternate with phases of depression with slow growth, and the position of the tumour in such a cycle at the time of the experiment would probably exert its influence.

Some of these possibilities were tested, and the results are recorded in later sections of this communication. Warburg postulates a disturbance of respiration as being the fundamental cause of the development of aerobic glycolysis. In many of the tumours included in the above table the respiration is very high, exceeding that of any tissue, normal or malignant, so far examined. In such cases, apparently, the Pasteur reaction fails to function, and until more is known of the mechanism by which respiration checks fermentation, the value of hypotheses involving conceptions of different kinds of respiration is doubtful.

The measurement of the respiratory quotient.

The Ringer solution used for the simultaneous measurement of respiration and aerobic glycolysis closely approximates, in its salt content and bicarbonate concentration, to physiological standards. The p_H is regulated by using 5 % CO_2 in O_2 as the gas phase, and for saturation of the media. The Henderson-Hasselbalch equation [Hasselbalch, 1917] then shows the p_H to be 7·35 at 37·5°. The procedure adopted for the measurement of the respiratory quotient involving absorption of CO_2 by potash is obviously inapplicable if these more accurate physiological conditions are to be maintained.

In the method utilised below, determinations of the respiratory quotient were made under the more favourable conditions used for simultaneous estimations of respiration and aerobic glycolysis.

Since Negelein [1925] has shown that the glycolysis effected by tumour tissue is a pure lactic acid fermentation and can be accurately measured by the fall in the bicarbonate concentration of the Ringer solution used experimentally, the respiratory CO_2 is obtained by deduction of the CO_2 evolved due to glycolysis (found by estimation of the bicarbonate concentration at the end of the metabolism experiment), from the total CO_2 evolved due to glycolysis and respiration and measured manometrically.

The following protocol will illustrate the method. In addition to the three manometers normally used for determination of aerobic metabolism, an extra one (Z) is required to measure the lowering of bicarbonate concentration during the 15 minutes' preliminary shaking until equilibrium is attained, *i.e.* to the time $t°$. This estimation, after correction for differences in weights of tissue used, serves as a control for the vessel Y in which the bicarbonate concentration is measured after 60 minutes' metabolism from the time $t°$. By rapid manipulation, the section of tissue can be removed from the Ringer solution in about 20 seconds, causing an error of no more than 0·5 %.

Protocol I.

Mouse tar carcinoma 173. Temp. = 37·5°. Gas phase = 5 % CO_2 in O_2.

	Vessel O	Vessel X	Vessel Y	Vessel Z
	Ringer thermo-barometric control $V_F = 3$ cc.	$V_F = 3{\cdot}5$ cc. $V_G = 4{\cdot}08$ cc. $K_{O_2} = 0{\cdot}365$ $K_{CO_2} = 0{\cdot}554$	$V_F = 1{\cdot}0$ cc. $V_G = 6{\cdot}28$ cc. $k_{O_2} = 0{\cdot}554$ $k_{CO_2} = 0{\cdot}608$	For bicarbonate estimation at $t°$ $V_F = 1{\cdot}0$ cc. $V_G = 5{\cdot}85$ cc.
Time	Dry wt. of tissues	3·85 mg.	3·73 mg.	5·18 mg.
$t°$	Shake 15′ to attain equilibrium	—	—	Section removed
$t° + 60'$	Shake for 60′	Increase of pressure $H = +43$ mm.	Increase of pressure $h = +57$ mm. Section removed	—
	Manometric estimation of bicarbonate concentrations	—	$B_2 = 487$ mm.³	$B_1 = 518$ mm.³

From the increases of pressure observed in the manometers attached to the vessels X and Y, the gas exchange is calculated in the usual way.

x_{O_2} = mm.³ O_2 consumed in vessel Y in 60′ = 27·6 mm.³

$x^{O_2}_{CO_2}$ = mm.³ CO_2 evolved (from glycolysis and respiration) in vessel Y in 60′ = 64·6 mm.³

The bicarbonate estimations are carried out with aliquot portions of the Ringer solutions from vessels Y and Z, together with a concurrent estimation of the bicarbonate concentration of the standard Ringer solution. The manometric method used is described by Warburg [1926]. As a check on the value obtained for vessel Y, the bicarbonate concentration in vessel X may similarly be determined. This was not carried out as a routine procedure since several such preliminary estimations for checking purposes showed it to be superfluous.

Expressing the bicarbonate concentrations as mm.^{3} CO_2 per cc. solution,

$B_0 = C_{NaHCO_3}$ of standard Ringer solution = 558 mm.^{3}

$B_1 = C_{NaHCO_3}$ at t° = 518 mm.^{3} (vessel Z).

$B_2 = C_{NaHCO_3}$ at $t^\circ + 60'$ = 487 mm.^{3} (vessel Y).

Therefore

$$\text{respiratory quotient} = \frac{x^{O_2}_{CO_2} - \left[B_0 - \frac{3{\cdot}73}{5{\cdot}18}(B_0 - B_1) - B_2\right]}{x_{O_2}}$$

$$= 0{\cdot}812.$$

Table II gives a series of the results obtained, with the simultaneously determined metabolism quotients.

Table II. *Simultaneous measurements of the carbohydrate metabolism and the respiratory quotient.*

Tumour	A Respiration Q_{O_2}	B Aerobic glycolysis assuming R.Q. is unity $Q^{O_2}_M$ (1)	C Aerobic glycolysis based on R.Q. found $Q^{O_2}_M$ (2)	D Anaerobic glycolysis $Q^{N_2}_M$	E Respiratory quotient
Tar carcinoma 173	−12·3	+10·8	+14·1	+33·4	0·735
,,	−10·2	+12·0	+14·2	+23·4	0·790
,,	− 7·4	+ 9·9	+11·3	+15·3	0·812
Tar carcinoma 2146	−19·2	+13·9	+ 9·7	+26·3	1·220
,,	−14·8	+11·7	+12·8	+20·6	0·772
,,	−15·2	+13·5	+14·7	+27·4	0·923
Crocker sarcoma	−11·7	+10·2	+12·1	+24·0	0·839
,,	− 8·8	+ 6·8	+ 8·5	+16·3	0·805
,,	−13·2	+10·6	+15·2	+22·8	0·649
Jensen's rat sarcoma	− 5·9	+ 9·4	+11·0	+21·6	0·722
,,	−12·2	+11·1	+13·6	+21·3	0·794
,,	−12·8	+14·5	+19·1	+26·5	0·638

The aerobic glycolysis has hitherto been derived by deducting the measured respiration from the measured total CO_2 evolved through the operation of the combined respiratory and glycolytic processes. The assumption has been made by Warburg and other workers with these methods that the respiratory quotient is unity, under the conditions of the manometric experiment. In column B the aerobic glycolysis is given, as reckoned on this basis.

In column C the aerobic glycolysis is calculated from the value of the respiratory quotient experimentally obtained. Since the respiratory quotients, with one exception, are less than unity, the aerobic glycolysis thus found is higher than that calculated on Warburg's assumption. The effectiveness of the respiration in checking glycolysis is consequently less pronounced than is indicated by the Meyerhof quotients in Table I.

Effect of glycolysis on respiration.

In connection with an investigation into the possibility of tumour tissue metabolising pentoses, results were obtained which suggest that the glycolytic activity of tumours may act as a partial check on their respiratory powers.

The metabolism values included in Tables III and IV show that tumour tissue does not utilise xylose. The magnitudes of the respiration, whether measured in media free from sugar or with xylose added, are almost identical. No splitting of xylose occurs, with formation of acidic products, either aerobically or anaerobically.

(*a*) *Respiration measurements.* These were carried out in Ringer solution containing 0·0025 mol. of sodium bicarbonate per litre. The initial p_H of the solution was approximately 8·2. Concentrated potash was used as absorbent for CO_2. Two series of tissues were used in these measurements; eleven transplantable animal tumours, and rat liver and kidney, representative of normal tissues.

Table III *a* summarises the findings with tumour tissues.

Table III *a*. *Respiration of tumour tissues in Ringer solution, with and without sugar additions.*

$C_{NaHCO_3} = 0{\cdot}0025$ mol. per litre. Gas phase O_2.

Tissue	Q_{O_2} Glucose added 0·2 %	Q_{O_2} Xylose added 0·2 %	Q_{O_2} No sugar added
Tar carcinoma 2146	−10·7	−12·1	−12·6
,,	−13·1	−16·6	−16·9
Jensen's rat sarcoma	−14·3	−16·6	−16·9
,,	−11·1	−13·9	−12·5
,,	− 9·8	−11·0	−11·1
,,	−10·4	−13·3	−13·1
,,	−12·1	−14·0	−14·3
,,	− 7·2	− 9·0	− 9·2
,,	−14·1	−16·7	−16·9
,,	−10·8	−13·1	−12·7
,,	− 9·7	−11·0	−11·2
Average	−11·2	−13·4	−13·4

With glucose-containing Ringer solution, the values obtained were consistently somewhat lower than those obtained in the absence of glucose.

The average lowering in the examples recorded was about 10 %.

Table III *b* shows the results obtained with normal tissues. Though the measurements were made under identical conditions, the effect of the addition of glucose was not to lower the respiration but, on the average, to raise it slightly.

Table III *b*. *Respiration of normal tissues, with and without sugar additions.*

$C_{NaHCO_3} = 0{\cdot}0025$ mol. per litre. Gas phase O_2.

Tissue	Q_{O_2} Glucose added 0·2 %	Q_{O_2} Xylose added 0·2 %	Q_{O_2} No sugar added
Mouse liver	− 8·8	− 8·2	− 6·6
Rat liver	−10·5	− 9·8	−11·1
,,	− 7·3	− 7·6	− 6·4
,,	−11·7	−12·0	−12·1
,,	− 9·2	− 8·9	− 8·9
,,	− 6·1	− 5·2	− 5·5
Rat kidney	−21·6	−20·3	−20·0
,,	−16·6	−15·0	−15·0
,,	−16·6	−17·6	−14·9
,,	−22·6	−22·2	−21·4
Average	−13·1	−12·7	−12·2

Since the arbitrary conditions under which these measurements of respiration were made deviate considerably from normal physiological conditions, particularly with respect to the p_H and the bicarbonate concentration, their general significance is doubtful. The implied checking of respiration by glycolytic activity was tested under more appropriate conditions, which are recorded in the following section.

(*b*) *General metabolism measurements.* The standard solutions for the simultaneous determination of respiration and aerobic glycolysis were employed, with $C_{NaHCO_3} = 0{\cdot}025$ mol. per litre and $p_H = 7{\cdot}35$.

Table IV gives a summary of the results obtained.

Table IV. *Comparison of tumour metabolism in presence of glucose and xylose.*

$C_{NaHCO_3} = 0{\cdot}025$ mol. per litre. Gas phase = 5 % CO_2 in O_2.

	Glucose added 0·2 %		Xylose added 0·2 %		
Tissue	Q_{O_2}	$Q_M^{O_2}$	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Crocker sarcoma	− 8·3	+12·1	−10·8	−	+1·2
,,	−10·6	+14·5	−11·4	−	+1·1
,,	−13·6	+15·5	−14·5	−	+1·6
,,	− 9·9	+16·8	−12·1	−	+0·8
,,	−15·8	+17·8	−15·8	−	+1·0
Jensen's rat sarcoma	−11·0	+ 9·3	−13·1	−	+1·3
,,	−11·6	+12·1	−13·7	−	+0·9
,,	− 9·8	+ 8·9	−11·2	−	+1·2
Average	−11·3	+13·4	−12·8	−	+1·1

Tissues from the same animal were used in concurrent estimations in the presence of glucose and of xylose. The results show an appreciable lowering of the respiration, about 12 % on the average, in the glucose-containing Ringer solution when compared with the xylose-containing Ringer solution. With xylose present no acid formation results through sugar splitting, either aerobically or anaerobically.

The tentative conclusion is that glycolytic activity exerts a significant checking effect on the capacity for respiration of tumour tissue.

Influence of environment on the carbohydrate metabolism of Jensen's rat sarcoma.

A few observations of the carbohydrate metabolism of Jensen's rat sarcoma were made with the intention of confirming the numerous consistent values found by Warburg and his collaborators.

Anomalous results were obtained, a tendency for the respiration to be high with respect to the anaerobic glycolysis being often noticed. The value of U was often negative. In Table V are collected the results of a few of these preliminary determinations.

The tumour strains of the Imperial Cancer Research Fund are propagated by subcutaneous grafts in the flank.

Table V. *Jensen rat sarcoma.*

Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Meyerhof quotient	U
−15·2	+13·5	+26·2	0·8	−4·2
−11·7	+10·2	+24·0	1·2	+0·6
−13·2	+10·6	+20·1	0·7	−6·3
−12·2	+11·1	+18·8	0·6	−5·6
−12·8	+14·5	+26·5	1·0	+0·9
−10·9	+ 9·3	+21·6	1·1	−0·2

The work of Campbell [1926] on tissue oxygen and carbon dioxide tensions has shown, by the method of gas injection, that the tensions of these gases vary in different parts of the body. Campbell and Cramer [1928] showed that rapidly growing implanted tumours in rats and mice show a greatly diminished rate of growth during prolonged exposure to low oxygen pressures. These observations suggested the possibility that the abnormal results recorded in Table V might be due to effects determined by the site of transplantation. Accordingly comparative series of metabolism measurements were made on two series of tumours, one series transplanted subcutaneously and the other intraperitoneally. The tumour used was Jensen's rat sarcoma, the rats chosen being of about the same age, 2 to 3 months. The inoculations were made with material from the same tumour and at the same date.

The tumours grown intraperitoneally are relatively more haemorrhagic than those grown subcutaneously and develop as a number of nodules adherent to neighbouring surfaces. As growth proceeds, these nodules unite, forming a large coherent mass. Isolated nodules provide the best material for metabolism experiments; microscopically they show numerous mitotic figures and no necrotic areas are visible.

The results are collected in Table VI.

Table VI. *Comparison of metabolism of Jensen rat sarcoma when transplanted subcutaneously and intraperitoneally.*

Subcutaneous transplantations				Intraperitoneal transplantations			
Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	U	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	U
−19·3	+12·3	+28·4	−10·2	− 6·2	+12·6	+31·6	+19·2
−16·1	+19·6	+32·6	+ 0·4	− 7·6	+11·9	+29·0	+13·8
−16·4	+16·8	+32·8	0	−12·3	+15·1	+26·9	+ 2·3
−13·6	+13·7	+27·6	+ 0·4	−11·1	+15·4	+34·0	+11·8
−18·9	+22·5	+31·6	− 6·2	− 8·6	+14·2	+25·4	+ 8·2
−19·3	+20·1	+33·4	− 5·2	−11·1	+15·0	+25·4	+ 3·2
−15·2	+14·1	+27·3	− 3·1	−13·3	+22·3	+37·5	+10·9
−16·6	+15·5	+27·3	− 5·9	−10·3	+22·2	+37·5	+16·9
−19·1	+25·8	+36·3	− 1·9	−12·3	+ 8·6	+25·0	+ 0·4
−27·9	+26·5	+36·4	−19·4	− 9·6	+ 8·4	+24·7	+ 5·5
−18·5	+24·5	+34·8	− 2·2	−12·4	+17·9	+33·6	+ 8·8
−14·2	+24·2	+34·9	+ 6·5	−12·9	+17·1	+32·8	+ 7·0
−15·3	+18·0	+30·6	0	−15·7	+17·2	+34·9	+ 3·5
−12·0	+17·5	+34·5	+10·5	−15·9	+17·2	+34·9	+ 3·1
−16·9	+23·3	+34·9	+ 1·1	−12·6	+17·4	+37·3	+12·1
−16·7	+25·4	+30·9	− 2·5	− 7·0	+18·4	+35·3	+21·3
−18·1	+25·2	+33·5	− 2·7	—	—	—	—
Av. −17·3	+20·3	+32·2	− 2·4	−11·2	+15·7	+31·6	+ 9·2

A consideration of these results leads to the following conclusions.

1. All the tumours grafted intraperitoneally show a carbohydrate metabolism conforming to that found by Warburg. A positive U, or excess fermentation, is a common property.

2. A large majority of the tumours grafted subcutaneously show a value for U which is either negative or near zero.

3. The magnitude of the respiration of the tumours grafted subcutaneously is considerably higher than that of the tumours grafted intraperitoneally. This increased respiration is in the neighbourhood of 50 %.

A corresponding increase in the aerobic glycolysis amounts to about 30 %.

4. The anaerobic glycolysis is, on the average, of the same magnitude in each series.

Summary and discussion.

1. Estimations of the carbohydrate metabolism of several strains of mouse tumours are recorded. Great deviations from the standard values found for tumours of rat, fowl and a limited series of human tumours were observed in many cases.

Wide variations are shown to occur between tumours of different strains, and also between members of the same strain.

The most noticeable feature is the number of cases of high respiration, both in its absolute value and also in its relation to the aerobic and anaerobic glycolysis. This respiration is ineffective in checking the aerobic glycolysis, its activity in this direction being, in some cases, less than 10 % of that found in the case of working muscle, and in many mammalian tumours.

Some factors which might operate in causing these variations are changes in the respiratory quotient, differences of environment during growth, efficiency of blood supply, and the generally higher metabolic rate of the mouse as compared with larger animals.

2. A manometric method for the simultaneous measurement of the carbohydrate metabolism and the respiratory quotient is briefly described, based on the fact that the glycolysis effected by tumour tissue is a pure lactic fermentation.

The respiratory quotients with one exception were found to be below unity. This would tend to make the actual aerobic glycolysis relatively higher than that usually recorded, since the assumption has hitherto been made that a respiratory quotient of unity would result from the experimental conditions.

The results again illustrate the ineffectiveness of respiration in checking glycolysis.

3. Xylose is not metabolised by tumour tissue.

4. Evidence is brought forward which suggests that the glycolytic activity of tumours exerts a checking effect on their respiration.

5. The carbohydrate metabolism of tumours is to some extent influenced by the environment in which they grow. This is demonstrated by the study of two series of Jensen's rat sarcomata, simultaneously transplanted, one series subcutaneously and the other intraperitoneally.

The respiration of the subcutaneous growths was, on the average, 50 % higher than that of the intraperitoneal growths. The majority of these subcutaneous tumours do not exhibit a positive value for the excess fermentation, which was, until recently, regarded by Warburg as a criterion for the metabolism of tumour tissue.

The correlation of these differences with the normal tissue tensions of CO_2 and O_2 is difficult. Campbell found the oxygen tension in the abdominal cavity 50 % higher than under the skin, the CO_2 tensions being approximately the same.

The higher respiration found in these two series of tumours corresponds to the lower O_2 tension in the surrounding tissues, and *vice versa*. It is obvious that other factors which have not yet been analysed are operative.

The general result of these observations is to emphasise the difficulty of including the wide variations found in the carbohydrate metabolism of tumour tissue in one generalisation.

The constant factor is the possession of a high aerobic glycolysis, which, though not specific for tumour tissue, is a source of energy available for uncontrolled proliferation.

The author is indebted to Dr J. A. Murray and other colleagues for transplanting the tumours used in these experiments.

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LXII. EXPERIMENTS ON NUTRITION. IX.

COMPARATIVE VITAMIN B VALUES OF FOODSTUFFS. PULSES AND NUTS.

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The good value of the "katjang-idjo" bean (*Phaseolus radiatus*) as a preventive and curative of beriberi was described by Grijns [1901]. Cooper [1912] and Chick and Hume [1917] subsequently made some quantitative tests with lentils and dried peas. The comparative values of these two pulses, with wheat germ as 100, was given in the Report on accessory food factors of the Medical Research Council (1924) as 80 and 40. Cajori [1920] found that the addition of 1 g. of various nuts to the diet restored growth to rats declining in weight. Observations by McCollum, Simmonds and Pitz [1917] on the nutritive value of the white bean led to the statement that 25 % of the bean in the diet of rats furnished sufficient vitamin B. Similar experiments with the soya bean by Daniels and Nichols [1917] and with the peanut by Daniels and Loughlin [1918] showed that 50 and 56 % of these foods supplied enough vitamin B for the rat. Also, Johns, Finks and Paul [1919] found that 75 % of coconut press cake had enough vitamin B for the rat.

In view of the scanty information about these foodstuffs in regard to their vitamin B content, and in order to obtain their comparative values more closely, we have undertaken this series of experiments. Our results must at present be considered as the vitamin B_1 + vitamin B_2 value, though the symptom of polyneuritis has been taken as far as possible as the criterion of the amount of vitamin in the foods. As our method of testing foodstuffs for vitamin B is very laborious the work has been limited to the more common varieties of pulses and nuts.

Experimental.

As in our former testing of foodstuffs for vitamin B [Plimmer, Rosedale, Raymond and Lowndes, 1927] pigeons have been used. They have been kept in pairs in suitably sized cages on the roof of the building. The earlier experiments were made with the foodstuff mixed with white rice and fish meal and with rearing as the standard of the vitamin B value. As the series of tests extended it was noticed that the birds scattered and separated the mixture

and so white flour was used in place of white rice. The ingredients were mixed into a dough and the dough divided into pills which were dried in the air, or at 37°. This mixture could not be separated, and thus the birds had to eat the mixture required for experimentation. The standard of rearing was considered too high and the standard of maintenance for at least 26 weeks was substituted. It had often been found that a period of 15 weeks was not long enough, and occasionally birds were observed to suffer from polyneuritis after as long a time as 30 weeks.

The result with each foodstuff is given shortly together with the times of the experiment and the weights, etc., in the tables.

Split peas.

In the first trial the birds were on the split pea diet for 106 weeks and showed no signs of any ill-effect. The other tests showed that for maintenance the diet must contain 30 % split peas. Rearing was possible on 40 %, but precarious, and not improved on raising to 50 %. Table I gives the details.

Table I. *Split peas.*

		Diet			Weights, g.			
Date	Time in weeks	Split peas	White rice	Fish meal	Cock No. 25/15	Hen No. 25/10	Number of eggs	Result
17. vi. 25		40	55	5	410	350	22 in Sept., Nov., Feb., Mar., Apr., May, June, July, Sept.	First 2 hatched and reared; 2 unfertile; 4 deserted; 9 fertile and deserted; 5 hatched and died in 2nd week
to								
12. x. 26	69				495	410		
13. x. 26		50	45	5			2 in Nov.	Both hatched and died in 1st week
to								
4. i. 27	12				515	435		
			White flour					
5. i. 27		40	55	5			10 in Feb., Mar., Apr., May	All fertile and deserted
to								
31. v. 27	21				480	390		
1. vi. 27		50	45	5			2 in June	Deserted
to								
28. vi. 27	4				485	430		
					No. 26/28	No. 26/30		
29. i. 27		30	65	5	415	505	16 in Mar., Apr., May, June, July	All were broken and deserted, some were fertile
to								
22. vii. 27	25				420	430		
					No. 21	No. 25/34		
20. i. 27		20	75	5	480	500	None	Both birds showed paralysis, but only hen seen with retracted head. Died
to								
13. ii. 27	4				310	315		
					No. 9	No. 10		
16. xii. 26		10	85	5	465	465	None	Both paralysed. Cock but not hen seen with retracted head. Died
to								
17. i. 27	5				390	290		

Whole dried green peas.

The first test was started at the same time as that with split peas. The pair were on the diet for 87 weeks altogether and on changing to white flour for another 15 weeks. The pair of birds, previously on 30 % split peas, after a period on brown rice, were given a diet with 30 % green peas to test for maintenance. They remained for 27 weeks in good condition. There seemed thus no difference between whole green peas and split peas and no further trial of green peas was then made. The tests with green peas at 20 % and 10 % were made later (Table II), and showed that 30 % dried green peas was the minimum for maintenance and that the husk contained no greater concentration of vitamin B than the endosperm.

Table II. *Whole dried green peas.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Green peas	White rice	Fish meal	Cock No. 25/1	Hen No. 25/13		
17. vi. 25 to 1. vi. 26	50	40	55	5	365	315	14 in Sept., Oct., Dec., Feb., Apr., May	6 hatched and reared; 4 fertile and deserted; 4 unfertile
2. vi. 26 to 28. ix. 26	17	30	65	5	410	350	15 in June, July, Aug., Sept.	All deserted; some fertile
29. ix. 26 to 15. ii. 27	20	40	55	5	450	360	4 in Oct.	All deserted
			White flour					
16. ii. 27 to 31. v. 27	15	40	55	5	490	400	12 in Feb., Mar., Apr., May	All deserted
1. vi. 27 to 28. vi. 27	4	50	45	5	390	350	4 in June	All deserted
					420	360		
					No. 26/28	No. 26/30		
14. ix. 27 to 21. iii. 28	27	30	65	5	410	435	10 in Oct., Feb., Mar.	2 hatched and died in 1st week; 8 deserted
					450	485		
					No. 96	No. 65		
10. x. 28 to 9. i. 29	13	20	75	5	395	405	None	
					375	385		
3. iv. 29 to 24. iv. 29	28				340	310		Hen died with large heart. Cock showed retracted head, and died
					300			
					No. 77	No. 128		
30. i. 29 to 13. iii. 29	6	10	85	5	495	445		
					320	320	None	Both showed paralysis, the cock with retracted head. Both died in 7th week

Lentils.

Altogether the pair of birds were kept 79 weeks on lentils and rice. There was an improvement in rearing on raising to 50 % lentils.

The trial with 20 % lentils was made on the birds previously used with split peas. They were given brown rice in the interval.

The details given in Table III show that for maintenance 30 % lentils are required. Rearing was scarcely possible on 40 %, but fairly good on 50 %.

Table III. *Lentils.*

Date	Time in weeks	Diet			Weights, g. Cock No.	Weights, g. Hen No.	Number of eggs	Result
		Lentils	White rice	Fish meal				
5. viii. 25 to 29. vi. 26	47	40	55	5	420	355	9 in Nov., Mar., Apr., May	2 unfertile; 3 hatched and died; 1 reared but abnormal; 1 dead at hatch; 2 fertile, deserted
30. vi. 26 to 8. ii. 27	32	50	45	5	420	440	7 in July, Aug., Sept., Oct.	2 hatched and died; 2 hatched and reared; 3 fertile, deserted
			White flour					
9. ii. 27 to 1. vi. 27	17	40	55	5	450 420	510 440	5 in Mar., Apr., May	All deserted. Birds escaped
					No. 75	No. 70		
9. ii. 27 to 22. vi. 27	19	30	65	5	430 380	435 425	6 in May, June, July	All deserted. Hen escaped
						No. 54		
6. vii. 27 to 25. viii. 27	7				395 410	340 370	2 in July	Deserted
					No. 25/15	No. 25/10		
18. viii. 27 to 12. x. 27	8	20	75	5	475 395	395 285	2 in Aug.	Deserted. Both birds showed retracted head and were cured

Haricot beans.

The first pair of birds did not like the beans and only ate them if compelled by not getting a new supply of food until the old had been cleared up.

The tests as given in Table IV showed that 40 % was required for maintenance.

The same pair, after a period of about 5 weeks on wheat, were started on 30 % haricot beans. At death both birds were found to have tumours.

Table IV. *Haricot beans.*

Date	Time in weeks	Diet: Haricot beans	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 25/14	Weights, g.: Hen No. 25/19	Number of eggs	Result
1. vii. 25		40	55	5	360	345		
to								
13. x. 25	15				230	315	None	Cock ill, chloroformed
to								
23. xii. 25	25					275		Hen ill, chloroformed
			White flour		No. 5	No. 4		
2. iii. 27		40	55	5	435	375	18 in Mar., Apr., May, June, July	Generally deserted after a few days, some found fertile
to								
10. viii. 27	23				440	395		
					No. 84	No. 37		
24. xi. 27		20	75	5	450	400		
to								
4. i. 28	6				320	320	None	Cock died with very wet tissues
to								
4. iv. 28	20					325		Hen died with typical polyneuritis
					No. 5	No. 4		
15. ix. 27		30	65	5	425	425	6 in Sept., Oct.	Deserted
to								
9. i. 28	17				475	340		Hen died with growth in oviduct
						No. 69		
29. ii. 28	24				445	305	9 in Mar., Apr., May, June	All deserted
to								
18. v. 28	40				305	340		Cock died; tumour in testis
to								
12. ix. 28	28					295		Hen showed signs of polyneuritis

Table V. *Soya beans.*

Date	Time in weeks	Diet: Soya beans	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 52	Weights, g.: Hen No. 42	Number of eggs	Result
8. ii. 28		30	65	5	490	530	10 in Apr., May, June, July, Aug.	All unfertile
to								
22. viii. 28	28				425	500		
					No. 100	No. 233		
24. iv. 28		40	55	5	410	425	4 in May, June	1 fertile; 3 unfertile
to								
27. vi. 28	9				415	475		
28. vi. 28		20	75	5			2 in July	Hatched and died
to								
7. xi. 28	19				345	370		Hen slightly paralysed and died; cock better
to								
20. i. 29	31				420			
					No. 37	No. 332		
17. x. 28		10	85	5	445	385	None	Both showed paralysis in 5th week; hen with retracted head
to								
14. xi. 28	4				315	315		

Soya beans.

The details in Table V tend to indicate that 20 % soya bean suffices for maintenance. On this quantity the birds began to lose weight, but slowly regained it. The hen in the 18th week suffered from an eye infection and died. Both birds ate very little during the time of the experiment and never looked really well. We have several times noticed that pigeons which eat little can maintain themselves on a diet with just too little vitamin B for a long time but not indefinitely. It cannot be considered that 20 % soya bean is enough; 30 % is nearer the minimum quantity.

Peanuts.

The test of peanuts is shown in Table VI.

20 % is enough for maintenance; rearing was possible on 40 %. Similar figures, twice as much vitamin B being required for rearing as is needed for maintenance, have been found in previous experiments.

Table VI. *Peanuts.*

Date	Time in weeks	Diet: Peanuts	Diet: White rice	Diet: Fish meal	Weights, g.: Cock No. 25/26	Weights, g.: Hen No. 43	Number of eggs	Result
26. ii. 26 to 29. ix. 26	31	40	55	5	455 430	430 415	8 in Mar., May, July, Aug.	5 hatched and reared; 1 hatched and died; 1 addled; 1 unfertile
30. ix. 26 to 9. ii. 27	19	30	65	5	490	485	1 in Oct.	Fertile, deserted
			White flour					
10. ii. 27 to 9. iii. 27	4	30	65	5	465	No. 23 450	4 in Apr., May	Hen escaped New hen. Fertile, deserted
to 25. v. 27	15				360	480		Cock not well; experiment stopped
					No. 83	No. 25/27		
23. ii. 27 to 24. viii. 27	26	20	75	5	410 390	385 350	16 in Feb., Mar., Apr., June, July, Aug.	3 hatched and died; 1 hatched and reared; 12 fertile, deserted
					No. 83	No. 25/27		
6. x. 27 to 14. iii. 28	23	30	65	5	415 435	390 280	10 in Oct., Nov., Dec., Jan., Feb.	2 hatched and died; 7 fertile; 1 unfertile, deserted. Hen suddenly not well; experiment stopped
					No. 88	No. 25/5		
16. vi. 27 to 31. viii. 27 to 2. xi. 27	11 20	10	85	5	395 360 325	405 325	2 in July	Unfertile, deserted Hen had polyneuritis and died Cock had polyneuritis and was cured with 1 g. of wheat germ ("bemax")

Almonds.

The vitamin B value of almonds was tested both with ground almonds, such as are used in making cakes, and with whole almonds, which were put through a mincer before being incorporated with white flour and fish meal. The two varieties were used to ascertain if the skin contained any vitamin B (see Table VII).

The first pair of birds were on the ground almond diet for 72 weeks in all. The other pairs on smaller amounts of ground almonds indicated that 40 % was required for maintenance.

The experiments with whole almonds (Table VIII) were started with 20 % and 30 % in the diet, the one with 40 % after the other results were known.

The minimum quantity of whole almonds for maintenance was thus 40 %, the same as with ground almonds. The skins do not appear to contain more vitamin B than the rest of the seed.

Table VII. *Ground almonds.*

		Diet			Weights, g.			
Date	Time in weeks	Ground almonds	White rice	Fish meal	Cock No. 1	Hen No. 5209	Number of eggs	Result
1. iv. 26 to 14. vii. 26	15	40	55	5	485 445	420 400	8 in Apr., May, June	2 unfertile; 6 fertile; no hatching
15. vii. 26 to 9. iii. 27	34	50	45	5		490	480	8 in July, Aug., Oct., Mar.	All fertile; 1 hatched and died
			White flour					
10. iii. 27 to 25. v. 27	11	50	45	5		470	410	6 in Apr., May	All deserted; some fertile
26. v. 27 to 24. viii. 27	12	60	35	5		470	440	8 in June, July, Aug.	All fertile, deserted
					No. 89	No. 49		
9. xi. 27 to 14. iii. 28	18	40	55	5	385 400	370 365	14 in Dec., Jan., Feb., Mar., Apr., May	Mostly fertile, no hatching Cock had head retraction in 17th week and was cured by 1 g. of dried yeast and remained well
to 20. vi. 28	32				380	350		
					No. 76	No. 449		
1. ix. 27 to 26. x. 27	8	30	65	5	435 425	485 410		Cock had head retraction in 9th week and died; hen ill, but recovered
to 8. ii. 28	19					430		
					No. 63	No. 92		
9. ii. 28 to 4. iv. 28	8	30	65	5	440 280	420 275		Both birds had head retraction and died
					No. 76	No. 16		
23. vi. 27 to 20. vii. 27	4	20	75	5	420 290	325 260		Both birds showed head retraction and were cured

Table VIII. *Whole almonds.*

Date	Time in weeks	Diet: Whole almonds	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 67	Weights, g.: Hen No. 128	Number of eggs	Result
15. iii. 28		30	65	5	380	425	2 in Mar.	Both hatched and died in 1st week with large hearts and wet tissues
to								
23. v. 28	10				300	380		Cock died 25. iii. 28 with paralysis, but head not seen retracted. Hen had head retracted on 29. v. 28 and was cured with 0·75 g. dried yeast
					No. 83	No. 294		
25. iv. 28		20	75	5	415	415	2 in Apr.	Deserted
to								
23. v. 28	5				355	285		Cock had head retracted in 5th week and was cured with 0·5 g. dried yeast. Hen had head retracted in 6th week and was cured with 0·5 g. wheat germ
					No. 2569	No. 50		
17. v. 28		40	55	5	345	470	2 in May	1 unfertile; 1 fertile, deserted
to								
31. x. 28	24				375	435		Cock escaped
to								
14. xi. 28	26					465		

Table IX. *Hazel nuts.*

Date	Time in weeks	Diet: Hazel nuts	Diet: White flour	Diet: Fish meal	Weights, g.: Cock No. 57	Weights, g.: Hen No. 6	Number of eggs	Result
7. xii. 27		30	65	5	520	475	6 in Mar., Apr., May	All fertile; 5 hatched and died
to								
13. vi. 28	27				475	395		
					No. 96	No. 65		
28. i. 27		20	75	5	380	425	8 in Mar., Apr., May, June	2 unfertile; 6 fertile, all deserted
to								
8. viii. 28	32				350	275		
to								
22. viii. 28	34				330	365		Hen showed polyneuritis; cured with 1 g. wheat germ
					No. 57	No. 6		
13. vi. 28		10	85	5	475	395		
to								
4. vii. 28	3				350	300		Both had polyneuritis and both cured with 0·5 g. marmite

Hazel nuts.

The test of hazel nuts is shown in Table IX. The pair on 30 % after a period of 27 weeks were then given 10 %. The minimum quantity for maintenance of

adult pigeons thus seemed to be 20 %. It may be noted that the hen became ill after 30 weeks and showed polyneuritis. This was possibly the result of laying so many eggs.

Chestnut.

For the purpose of these experiments dried chestnuts were used, as they could be obtained during almost the whole year, and as they were in this state more easily comparable with almonds and hazel nuts.

As a preliminary to the trial a pair of birds were given the dried chestnut with 5 % fish meal to see if they would eat it. It was eaten well and the birds increased in weight. In the first trial the birds were on the chestnut diet for 75 weeks. They were then given a diet of brown rice with 5 % fish meal and later tried with 30 % chestnut. Table X shows that the quantity of dried chestnut for maintenance is 40 %. Some rearing was possible on this amount, but it was better with 50 %.

Table X. *Chestnut.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Chest-nut	White rice	Fish meal	Cock No. 7	Hen No. 8		
10. ii. 26 to 3. iii. 26	3	95	0	5	500	385		
					555	445		
4. iii. 26 to 9. vi. 26	14	50	45	5	465	380	4 in Mar., Apr.	All hatched and reared
10. vi. 26 to 16. iii. 27	40	40	55	5	505	425	14 in June, July, Aug., Sept., Feb.	2 hatched and reared; 3 unfertile; 9 fertile, deserted
			White flour					
17. iii. 27 to 1. vi. 27	11	40	55	5	445	405	10 in Mar., Apr., May	All deserted
2. vi. 27 to 10. viii. 27	10	50	45	5	455	385	5 in June, July	All deserted
			Brown rice					
11. viii. 27 to 5. x. 27	8	0	95	5	510	430		
			White flour		No. 14	No. 13		
24. viii. 27 to 20. ix. 27	4	20	75	5	450	395	2 in Aug.	Deserted
					335	285		Cock showed retracted head. Cured with 1 g. wheat germ
26. x. 27	10					260		Hen had paralysis and died
					No. 7	No. 8		
6. x. 27 to 23. xi. 27	7	30	65	5	420	340	2 in Oct.	Deserted
								Cock showed retracted head. Cured with 1 g. of wheat germ
30. xi. 27	8				420	330		Hen showed retracted head. Cured with 1 g. of wheat germ

Coconut.

The coconut used in these experiments was the material commonly employed for making cakes and sweets, and consisted of the dried and flaked flesh of the nut.

It was expected that this food would show about the same value as the other nuts, and the first trial was made with 40 % mixed with white rice. The birds did not appear to eat the coconut, and after 6 weeks the trial was stopped as the birds had lost weight and looked ill. They were put upon millet, their former diet, for the next 3 weeks and were then given coconut mixed with 5 % fish meal. The food was not liked and little was eaten. Both birds showed paralysis and died.

Table XI. *Coconut.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Coco-nut	White rice	Fish meal	Cock No. 15	Hen No. 16		
27. i. 26 to		40	55	5	495	430		
10. iii. 26	6				340	360		Ate mostly rice
31. iii. 26 to		95	0	5	405	425		
28. iv. 26 to	4				335	355		Cock showed retracted head and died
23. vi. 26	12					280		Hen showed paralysis and died
					No. 25/14	No. 25/27		
2. vi. 26 to		95	0	5	365	345		
7. vii. 26 to	5				290	285		Cock showed retracted head and died
21. vii. 26	7					245		Hen showed retracted head. Cured with 1 g. yeast

Table XII. *Coffee.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Green coffee	White rice	Fish meal	Cock No. 7	Hen No. 8		
5. i. 28 to		30	65	5	600	445		
29. ii. 28 to	8				345	305		Cock showed retracted head. Cured with 1 g. wheat germ
21. iii. 28 to	11				325	285		Cock died
28. iii. 28	12					255		Hen was paralysed and died
		Roasted coffee			No. 39	No. 50		
29. xii. 27 to		30	65	5	380	455		
1. ii. 28	5				225	305		Both birds died
					No. 84	No. 62		
10. ii. 28 to		50	45	5	370	385		
9. iii. 28	4				245	255		Both birds died

As coconut is such a common foodstuff one more trial was made. The former result was confirmed (see Table XI).

The flesh of the coconut, as prepared for making cakes, thus appeared to contain no vitamin B.

Coffee.

It was not expected that roasted coffee would contain vitamin B, but it was felt necessary to try the experiment. Two experiments were made with 30 and 50 % coffee. The birds died after the 5th and 4th weeks respectively.

At the same time a trial was made with 30 % green coffee. As the birds lived for 8 to 12 weeks it would appear that green coffee contained some vitamin B. A higher proportion in the diet was not tried. The vitamin in the seed is thus destroyed during the roasting (see Table XII).

Discussion and summary.

Previous work upon the vitamin B value of pulses and nuts suggested that the pulses had varying values, but that nuts were of more uniform value. Our results do not support the older observations. Pulses have been found to have a very similar vitamin B value, whilst nuts showed considerable variation. Hazel nuts were of highest value, coconut was of no value.

It is convenient to adopt a standard for comparison. In our former work [1927] dried yeast was taken as standard. 4 % of this food was found sufficient to maintain a pair of pigeons for 26 weeks or longer. It was placed at 100. Using the same standard the pulses and nuts now tested come out as follows.

	Percentage amount in diet for maintenance	Relative vitamin B value
Dried yeast	4	100
Split peas	30	13
Whole dried green peas	30	13
Lentils	30	13
Haricot beans	40	10
Soya beans	30	13
Peanuts	20	20
Ground almonds	40	10
Whole almonds	40	10
Hazel nuts	20	20
Dried chestnuts	40	10
Coconut	No maintenance	0
Coffee, green	Over 30	Less than 13
Coffee, roasted	No maintenance	0

These figures do not compare strictly with those given for cereals in our former work, as the standard of hatching and rearing was then taken as criterion of the vitamin B value. The values of cereals are being re-determined on the standard of maintenance for a period of at least 26 weeks. The amount of whole wheat for maintenance has been found to be 40 %, whilst oatmeal and maize are showing 50 %, a higher value than was expected. Other tests are being made with fruits and vegetables. Potatoes and artichokes

at 80 % and parsnips and leeks at 60 % of the diet have been found to maintain pigeons for long periods. The other vegetables and fruits have no appreciable quantity of vitamin B.

In these experiments it has again been observed that for hatching and rearing of young pigeons more vitamin B is required than for maintenance. In many cases rearing has been possible on double the amount needed for maintenance.

As it is not possible at present in the case of these foodstuffs to differentiate vitamin B into B_1 and B_2 factors, the results must be considered as due to the two factors together, though the symptoms of polyneuritis have been taken as the indication of the value. Loss of weight cannot be regarded as an indication of vitamin B_2 value. As soon as the food mixture is not suitable for maintenance of the pigeons, consumption of the food gets less and less and the birds lose weight in consequence. Polyneuritis has never been observed without refusal of the birds to eat. Both Cooper, and Chick and Hume, have mentioned quantities of foodstuffs required to prevent loss of weight of pigeons, but our experience indicates that continuation of their experiments for a longer period would have led to polyneuritis.

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LXIII. FURTHER OBSERVATIONS ON THE EFFECTS OF LARGE DOSES OF IRRADIATED ERGOSTEROL.

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Recent observations on the effects produced in animals by excessive feeding with irradiated ergosterol have provided definite results which are at variance. Pfannensteil [1927], Kreitmair and Moll [1928], and Harris and Moore [1928] have shown that rats and some other animals become ill in a few days and die in a week or two when they are given some milligrams of this substance daily in addition to a normal diet; and Kreitmair and Moll [1928] and Kreitmair and Hintzelmann [1928] described an atrophic spleen, haemorrhagic enteritis, and extensive and severe arteriosclerosis as the prominent pathological features. On the other hand, Dixon and Hoyle [1928] were unable to confirm these results. They found that rats failed to gain in weight when fed with similar amounts of irradiated ergosterol, and when killed, after being fed in this way for about 40 days, showed calcium phosphate calculi in the urinary tract. Dixon and Hoyle used ergosterol which had been irradiated in oily solution, and they suggested that the medium in which irradiation is effected may be a deciding factor in determining the toxicity of the product as shown by feeding experiments with very large doses. In the experiments of Harris and Moore the ergosterol had been irradiated in alcoholic solution. The German observers did not state their method of irradiation.

In the experiments described in the present paper the work of Dixon and Hoyle has been repeated with the addition of certain quantitative estimations on the urine in an attempt to determine the immediate cause of the formation of the calculi found in such experimental animals.

Experimental.

Twenty-four half-grown albino rats each weighing about 180 g. were chosen for the experiment and kept throughout in cages of the Hopkins pattern, two rats in each cage. The urine was collected by Hopkins's method in 3-day periods, and that from three cages mixed and analysed together. In this way four groups, each consisting of six rats, were maintained, and two of these groups received the experimental diet and two were kept as controls. The

volume of urine passed by each group was measured separately. The contents of the three flasks from each group were poured into a graduated cylinder and the volume noted. In this way the average amount of urine passed per rat per day could be calculated. The urine was then poured back into one of the flasks; the graduated cylinder and the two remaining flasks were washed out with 20 cc. of distilled water and these washings were added to the main bulk of urine. The analyses were made on this diluted urine and allowance made for the dilution by calculation. In the earlier estimations the urine was acidified with 5 cc. of *N* HCl in order to dissolve any calcium phosphate which might have crystallised out: in the later estimations, which were made daily, no acid was added. When the estimations were not carried out immediately the urine was preserved with chloroform.

We intended to estimate the urinary calcium excretion, but we found that in using the ordinary Shohl and Pedley technique [1922] for the calcium estimations it was impossible to obtain with certainty a sufficiently clear solution after digesting the urine with ammonium persulphate. In the case of rats' urine this oxidation gives rise to an insoluble substance which has not yet been thoroughly investigated. As it was partly organic in nature, we considered that further investigation of the completeness of the oxidation of urates and allied substances in rats' urine by this method was necessary before it could be relied upon as a step in the estimation of calcium. For this reason we were compelled to abandon calcium estimations in the present experiments.

Urinary phosphate was estimated by Briggs's method [1922]. The acidified urine was shaken and allowed to stand; 10 cc. were withdrawn and the HCl previously added neutralised by the calculated amount of *N*/10 NaOH. The solution was then made up to 100 cc. and 1 cc. used for the estimation. During the last 9 days of the experiment daily estimations of urinary phosphates, chlorides, and p_H were made. Chlorides were determined by Whitehorn's modification of Vollhard's method. The hydrogen electrode was used for the p_H determinations. In all the estimations duplicates were done as far as possible, and gave satisfactory readings.

All the animals received a control synthetic diet for a preliminary period of 14 days. The synthetic diet consisted of caseinogen 20 %, potato starch 35 %, sugar 10 %, cacao butter 20 %, orange juice 5 %, marmite 5 %, salt mixture 5 %. The salt mixture was that given by Hume and Smith [1928], with the addition of 0·1 % potassium iodide and traces of sodium fluoride and manganese sulphate. All the animals received in addition to this diet 2 drops of a standardised cod-liver oil daily. The experimental diet contained 1 % of irradiated ergosterol in the cacao butter so that 10 g. of the completed diet contained 20 mg. of irradiated ergosterol. The irradiated ergosterol, kindly supplied by the British Drug Houses, was irradiated in oil as described by Dixon and Hoyle [1928]. Distilled water for drinking was supplied in ample quantity in glass bulbs. After the preliminary control period, half the animals were given the experimental diet, and the other half continued on the control

diet. The experimental period was continued for 45 days. In all cases the diets were given mixed into a stiff paste with a little distilled water in order to reduce scattering to a minimum. Each rat was allowed 15 g. of the dry diet daily, and the leavings from each cage were weighed each day. In this way the average quantity of food and irradiated ergosterol taken per rat could be accurately estimated.

In the urinary estimations there are two main sources of probable error, contamination of the urine with faeces and with food. It is impossible to prevent faecal particles adhering to the urine collectors occasionally; and it is impossible entirely to prevent scattering of food. In order to decide the extent of the errors involved in these ways, two estimations were made. In the first case four faecal masses were shaken up for 5 minutes with 100 cc. of distilled water. After sedimentation 1 cc. of the fluid was withdrawn for the estimation of phosphate. The amount of soluble phosphate present, however, was so small that no detectable blue colour was produced with the Briggs reagents. In the second place 2 g. of control diet were shaken up with 100 cc. of distilled water. The phosphate in this solution was then estimated and it was found that 5·5 mg. had passed into solution. Supposing that 50 cc. of urine are contaminated with 2 g. of diet, then approximately 11 mg. of phosphate are added; this is equivalent to an error of about 3 % and is negligible when the additional sources of error that are inseparable from such animal experiments are considered. But it is extremely improbable that so large a quantity of food as 2 g. was ever mixed with the urine.

Results.

All the control rats throughout the entire experiment and the experimental rats during the preliminary control period maintained an approximately steady food consumption. When the experimental rats began to take the diet containing the irradiated ergosterol they showed a decrease in appetite during the first week. During this time they lost weight to a corresponding degree and although subsequently they returned to their normal level of food consumption this was not accompanied by a gain in weight. During the remaining period of the experiment the average weight curve for the twelve experimental animals remained practically steady. This is shown in Fig. 1. The average amount of irradiated ergosterol taken per rat per day over the whole experimental period was 26·4 mg.

The daily output of urine and the urinary phosphate excretion for the control and experimental groups of rats are shown in Figs. 2 and 3 respectively. The figures for both control and both experimental groups have been averaged and presented together since there were no important differences between the two sub-groups. It will be seen that the urine output of the control group was constant within definite limits throughout the whole experiment. The daily urinary phosphate excretion varied, however, considerably from day to day and is throughout placed at a very high level relative to that of other animals.

During their control period the experimental animals were strictly comparable with the controls both as regards their urine output and urinary phosphate excretion. Within 3 days of the administration of the irradiated ergosterol these animals developed a considerable diuresis which persisted throughout the subsequent 42 days of the experiment. During this time the extent of the diuresis remained roughly constant and represented an increase of 200 to 300 % on the previous water excretion. The urinary phosphate passed by these animals per rat per day remained almost at its previous level, though still showing considerable daily fluctuations.

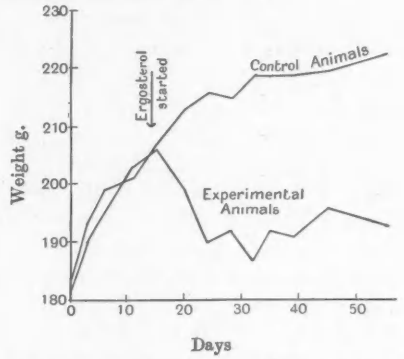

Fig. 1. Showing the average weight curves of the control and experimental animals.

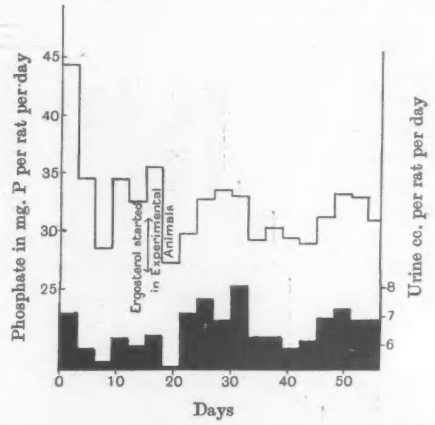

Fig. 2. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 control animals.

During the last 9 days of the experiment urinary chloride and p_H determinations were made. The figures in Table I show that the total chloride and phosphate passed per rat per day during this period by the control and

experimental groups show no significant differences. The average values of the p_H determinations were also similar.

Table I.

The figures in column 1 are the arithmetic means of the individual readings.
Column 2 is calculated from the formula used by Hill, Long and Lupton [1924].
Column 3 expresses as a percentage the largest observed discrepancy between an individual reading and the mean.
Column 4 expresses as a percentage ratio the difference between the mean values for the control and experimental animals.
Phosphate expressed in mg. P; chlorides expressed in mg. Cl.

		1 Mean per rat per day	2 Probable percentage error of mean (calculated)	3 Maximum percentage error of mean (observed)	4 Percentage deviation of experimental mean from control mean
Urine	Controls	7·06 cc.	2·8	21	
	Experimentals	17·8	3·3	26	151
Phosphate	Controls	32·5 mg.	2·4	17	
	Experimentals	38·0	4·8	32	17
Chlorides	Controls	44·0 mg.	3·8	23	
	Experimentals	52·8	3·6	26	17·5
p_H	Controls	6·21	3·5	31	
	Experimentals	6·5	3·2	26	4·7

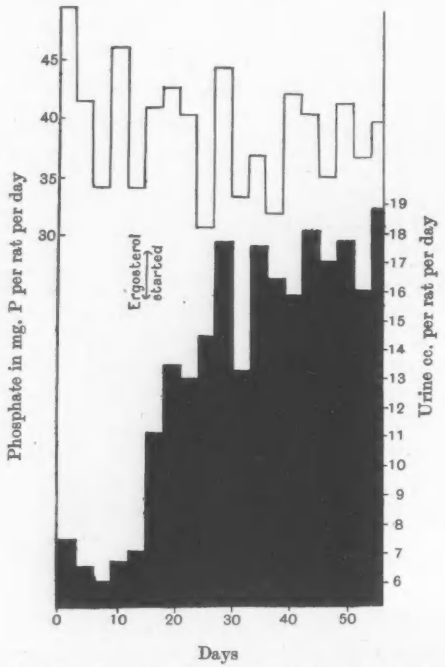

Fig. 3. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 experimental animals.

Towards the end of the experiment several of both the control and experimental rats were X-rayed; only in one of the experimental animals was a calculus defined with certainty, and in this case a calculus, some 4 mm. in diameter on the plate, was observed in the bladder. At the end of the experiment all the rats were killed and examined *post mortem*. No pathological changes were observed in any of the control animals. In all the experimental rats the kidneys presented a streaky, yellowish white appearance at the corticomedullary junction, in some cases extending into the papilla, similar to that already reported by Dixon and Hoyle. In nine of these rats calcium phosphate calculi were found in the kidneys, ureters or bladder. They varied in number, size and position, but the frequency of secondary mechanical changes was not so marked as in the former experiments of Dixon and Hoyle. In no case was there evidence of gross infection of the urinary tract. In four animals there was some degree of arteriosclerosis of the thoracic or abdominal aortae. In all these cases the other main arteries showed no lesions.

Discussion.

This experiment affords complete confirmation of the results already published by Dixon and Hoyle. In no case did the irradiated ergosterol administered prove fatal to the experimental animals, and the weight changes and *post mortem* findings confirm their results except in the presence, in four of our experimental animals, of some degree of arteriosclerosis in the aorta. As this was present in a few animals only, and was limited in distribution even in these, and as further the experiment had been continued about four times longer than in the case of those reported by the German workers, it is evident that the arterial changes found in the present case have considerably less significance.

All the experimental animals showed a persistent diuresis without any significant increase in either the total phosphate or chloride output in the urine, so that the concentrations at which these substances were passed varied inversely to the extent of the diuresis. As there was no increase in the total phosphate output this substance was not the cause of the diuresis, since, with a low threshold substance such as this, a diuresis directly due to it causes an increase in total output without any marked fall in the concentration at which it is passed. No further light was thrown on the matter by the p_H determinations. The average urinary p_H of the experimental animals was slightly more alkaline than that of the controls, as would be expected with a diuresis. The figures show that there is no evidence that phosphate is precipitated in the urinary tract as the result of a marked change of p_H towards the alkaline side. Moreover, during the diuresis the total outputs of phosphate and chloride, and the relative concentrations at which they were passed, followed an approximately parallel course. This shows that there is no evidence of any selective effect on the tubular reabsorption of phosphate.

The cause of the diuresis remains at present unexplained. It may well be that a marked increase in the urinary calcium excretion is the determining factor. If this is not so, the occurrence of urinary calculi composed of calcium phosphate in animals that have exhibited a diuresis in which the urinary phosphate concentration is always less than half the normal, without any increase in the total phosphate passed and without any change in the reaction of the urine, must be a very unusual condition.

Conclusions.

1. Confirmation of the effects described by Dixon and Hoyle as occurring in rats given large doses of irradiated ergosterol have been obtained. Emphasis is laid on the non-lethal effects, the absence of persistent or marked loss in weight, and the presence of urinary calculi *post mortem* as the main features of such experiments when ergosterol that has been irradiated in oily solution is used.

2. Such animals show a persistent diuresis without any increase in the total phosphate and chloride passed per day, and without any significant change in the reaction of the urine.

The work in this paper has been done during the tenure by one of us (J. C. H.) of the Ernest Hart Memorial Scholarship of the British Medical Association.

Note (added 11. v. 29, Ed.).

In a paper published since this was sent to press, Harris and Moore [1929] have confirmed their previous findings with ergosterol irradiated in alcohol, and have further found similar effects with ergosterol irradiated in oil. In both cases the *post-mortem* findings were identical with those described by the German workers, with in addition atrophy of the thymus, and in many cases the presence of urinary calculi. Further, they found that ergosterol which had been over-irradiated was completely innocuous, and on the basis of these results they consider that the suggestion made by Dixon and Hoyle, that some additional toxic factor dependent upon the method of irradiation is responsible for poisonous effects, is not valid. If their findings are confirmed this must be so. But in the experiments described in our paper no such results have been found.

Harris and Moore suggest that the reason why Dixon and Hoyle failed to obtain the toxic effects in full measure is because insufficient irradiated ergosterol was given, or that it was not administered for a sufficient length of time. We do not think that this can be the explanation. In the first place, Harris and Moore have obtained the full toxic effects in about 40 days when the equivalent of only 1 to 2 mg. of ergosterol irradiated in alcohol was given daily, as "radiostol," *i.e.* irradiated in oil. Assuming that this ergosterol was of maximal antirachitic activity—that is, about 10,000 units per mg.—the daily dose would be some 20,000 antirachitic units. In Dixon and Hoyle's experiments up to

17 mg. daily were given, and in the experiments in the present paper an average of 24 mg. daily, in both cases for the same period of time as in Harris and Moore's experiments. Although this particular sample was not tested for antirachitic activity, samples irradiated under exactly similar conditions for the same length of time have shown an antirachitic activity of 1600 units per mg. In our experiments, therefore, some 30,000 to 40,000 units at least were given daily. As the samples used by Harris and Moore were irradiated under similar conditions to ours it is improbable that they were of maximal antirachitic activity, and although these writers state that they were assayed they do not give the values found.

The cause of the difference in experimental findings has yet to be determined.

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LXIV. THE EFFECT OF PARTIAL DECAY ON THE ALKALI SOLUBILITY OF WOOD.

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Introduction.

It is generally accepted that at least two types of wood decay are caused by fungi, namely the "brown" and "white" rots. In the former type preferential attack is made on the carbohydrate components of wood substance, and lignin is in the main unaffected, the decayed residue being usually brown in colour. In the latter type lignin seems to be the main objective of the fungus, and in the decayed residue there remain patches or "pockets" of a white substance reputed to consist of pure cellulose. The type names are thus derived from the colour of the residues after attack.

In brown rots it has been shown by the work of Bray and Andrews [1924] that the causal fungi are consistent in their preferential attack on the carbohydrate components, but it cannot be said of the white rots that they are consistent in that lignin is always attacked in preference to carbohydrates. Only a few fungi of the white rot type have been studied in any detail, however, but Hawley and Wise [1926, 1] point out that while one of them, *Trametes pini*, may show a preference for lignin, another, *Polyporus hirsutus*, has a serious effect on the cellulose of the wood of white spruce. It is clear, therefore, that the terms "brown" and "white," as applied to different types of decay, can be accepted in an approximate sense only, since inconsistencies are encountered, in the case of the white rots at least, when the chemical effects on the host are taken into consideration.

A classification of decay types based on the chemical effects of the fungus on the host is suggested in the work of Falck and Haag [1927], Wehmer [1927], and Bavendamm [1927]. These authors favour the use of the terms "destruction" and "corrosion" respectively, the former type to include all forms of fungal decay in which carbohydrates are preferentially attacked, and the latter to include the forms in which lignin is preferentially attacked. According to Wehmer [1927], the "destruction" type is brought about by hydrolysis of carbohydrates, and in the "corrosion" type lignin is depleted by a process of oxidation. Hawley and Wise [1926, 2], however, are not disposed to consider decay purely as one chemical process, on the grounds that any decomposition of a nature so profound as the fungal decay of wood could not strictly be compared to a single type of chemical reaction.

In much of the work already done on the chemistry of decay, material has been examined in which decay has been allowed to proceed to a considerable extent, and there is little doubt that in its advanced stages fungal decay is a profound decomposition of a nature difficult of diagnosis by present methods of analysis. At the same time, however, there is evidence that decay takes place in stages [Hawley and Wise, 1926, 3], and the possibility must not be overlooked that, in the very early stages, reactions of a comparatively simple nature may be involved. Also, for all practical purposes the initial stages of decay are the most important, since it is against these that all preservative treatments must be applied. It is obvious, therefore, that a closer understanding of the mechanism of incipient decay should lead to better methods in wood preservation.

The one outstanding feature with regard to fungal decay in wood is that, as a direct result of infection, the residue is rendered more soluble in sodium hydroxide than the original sound wood. In fact Bray [1924] has pointed out that in the case of the brown rots, increase in alkali solubility serves as an index of the extent of cellulose depletion in any given sample of decayed wood. It has also to be noted that acid hydrolysis has the effect of increasing the alkali solubility of wood, and this fact has led Hawley and Campbell [1927] to conclude that there must exist a relationship between acid hydrolysis on the one hand and fungal decay on the other. That fungi are capable of producing acid in various media has been shown by Curtin [1927], and this further strengthens the contention of the above authors, since in the course of its action on wood the fungus may produce acid whereby hydrolysis is brought about. The present investigation has been undertaken in order to obtain a closer comparison between acid hydrolysis and decay than has hitherto been available.

Experimental.

The wood used was Sitka spruce heartwood sawdust of 60–80 mesh. The analytical methods were those recommended by Schorger [1926] and Hawley and Wise [1926, 4].

The general scheme employed was the same as that used by Hawley and Campbell [1927] in their study of acid hydrolysis. Analytical data were obtained for the main constituents of the original wood, and the effect of 1 % sodium hydroxide at 100° was determined on these as before. The results are given in Tables I and II.

Table I. *Analysis of original wood.*

Results on basis of weight of original dry wood.

Cold water-soluble	1·6 %
Hot water-soluble	3·6
1 % alkali-soluble	12·6
Cellulose	62·6
Lignin	26·2
Methoxyl	4·7
Total pentosans	8·4
Pentosans not in cellulose	5·1

Table II. *Analysis of residue after treatment with* 1 % *NaOH for* 1 *hour at* 100°.

Results expressed as percentages by weight of original dry wood.

Loss on alkali treatment	13·8
Cellulose	59·0
Lignin	24·3
Methoxyl	4·0
Total pentosans	6·8
Pentosans not in cellulose	3·9

In studying the effect of decay on alkali solubility, samples of decayed wood were analysed, treated with sodium hydroxide, and the residues analysed. The experiments were carried out in the following manner.

Three separate weighed samples of the 60–80 mesh material were placed in flasks, and moistened with water. The flasks were then plugged with cotton wool and sterilisation was effected by steaming for 30 minutes on each of three consecutive days. After inoculation with *Trametes serialis* Fr. one sample was left to decay for 3 months, and the other two for 4 months at 20°.

After decay each sample was collected on a linen filter and washed with cold water until free from acid. Drying was effected by suction as far as possible, and then, to arrest decay, the samples were further dried and sterilised by heating in weighing bottles at 105° for 16 hours. After cooling in desiccators the samples were weighed, and the loss in weight sustained by each was determined.

Each sample was now divided into two portions, one to be analysed without delay, and the second after treatment with 1 % sodium hydroxide for 1 hour at 100°. The analytical results are given in Tables III and IV.

Table III. *Analysis of residues after decay and washing with cold water.*

Results expressed as percentages by weight of original dry wood.

Duration of decay	Loss due to decay plus cold water washing	Hot water-soluble	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose
3 months	6·98	2·3	55·5	25·8	4·3	7·2	4·8
4 months (1)	8·0	2·9	53·6	25·9	4·2	6·9	4·7
4 months (2)	8·96	3·4	52·7	25·9	4·2	6·8	4·7

Table IV. *Analysis of residues after decay, washing with cold water, and subsequent treatment with* 1 % *NaOH for* 1 *hour at* 100°.

Results expressed as percentages by weight of original dry wood.

	Loss due to decay plus cold water washing	Loss on alkali treatment	Total loss	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose
(1)	6·98	23·1	30·08	44·7	22·6	3·6	4·6	2·6
(2)	8·0	22·4	30·3	42·9	23·0	3·5	4·4	2·4
(3)	8·96	24·9	33·86	40·0	22·5	3·5	4·1	2·4

Discussion.

As previously mentioned a steaming treatment was applied prior to inoculation of the wood with the fungus. That such a treatment has no detrimental effect on spruce wood has been shown by Hawley, Fleck and Richards [1928].

The results in Table III were obtained after the decayed residues had been washed with cold water and heated for 16 hours at 105°. These steps were considered necessary for the following reasons.

In the first place the decayed residues were found to be distinctly acid, hence it was imperative that all traces of acid should be removed prior to analysis. The loss due to decay and the cold water-soluble material was therefore determined as a whole. That some water-soluble material remained in the residues after the preliminary washing was proved by carrying out a hot water-soluble determination on each sample. Therefore, although the exact loss due to decay itself has not been determined, its effect on the wood can be visualised by a comparison of Tables I and III, for, even if all the water-soluble material had been left in the residues, it is certain to have been lost during subsequent analysis, and thus the analytical data would have been the same as those recorded.

As to the heat treatment applied after washing and prior to analysis, this was considered to be the only efficient means of arresting decay at the required time, and of getting the material into a convenient state for analysis. Peterson and Bray [1928] have pointed out that an oven-drying treatment has the effect of lowering cellulose yields, and of rendering the isolation of cellulose more difficult, but in this investigation no difficulties were experienced in isolating cellulose with a normal amount of chlorination after heating the residues for the time stated.

Fungal mycelium, if present in considerable amount in decayed residues, would tend to vitiate analytical results, but attempts to apply corrections for this source of error have proved unsatisfactory. In any case, where decay is only allowed to proceed for a short period, the actual amount of mycelium in the infected material could not be sufficient to detract from the purely comparative value of the analytical data. Care was taken thoroughly to mix the decayed material so that mycelial remains should be distributed as evenly as possible throughout the whole.

Comparison of Tables I and III shows that the fungus has behaved as a typical brown rot in the manner of its attack on wood. Cellulose and pentosans have been depleted, and lignin has been affected to a slight extent, the comparatively small loss in lignin being consistent with a corresponding fall in the methoxyl content. Moreover, it can be seen that the pentosan losses are chiefly due to depletion of the pentosans in the cellulose. That such behaviour is typical of brown rots has been amply demonstrated in previous work, but it was necessary to obtain these results for comparison with Table IV which

shows, firstly, the marked increase in alkali solubility due to decay, and secondly, the detailed effect of alkali treatment on decayed wood.

The increase in alkali solubility is largely accounted for by losses in cellulose and pentosans, the lignin in decayed wood being only slightly more soluble in alkali than the lignin in sound wood.

Whereas in Table I the sum of the four main constituents—namely water-soluble material, cellulose, lignin, and pentosans not in the cellulose—amounts to 99·1 % of the original wood, the sums of the main constituents in the samples of decayed wood, inclusive of the losses due to decay and water-soluble material, amount to 95·58, 95·1, and 95·66 % of the original wood respectively. These facts are in agreement with previous work in that they illustrate that there is in decayed wood a material which is insoluble in water, and is not determined as cellulose, lignin, or pentosans. That such material is probably a carbohydrate degradation product is illustrated by the fact that only the carbohydrates in the wood have been seriously affected by decay. Further, in Table IV, the sums of the main constituents, including the losses due to decay and alkali treatment, amount to 99·98, 98·7, and 98·76 % respectively of the original wood. The fact that these totals are approximately equal to the sum of the main constituents in the original wood indicates that, whatever the material undetermined in the decayed wood may be, it is largely soluble in 1 % sodium hydroxide.

Conclusions.

In comparing the foregoing experimental data with regard to decay and its effect on the alkali solubility of wood with those cited by Hawley and Campbell [1927] in their study of acid hydrolysis, a marked similarity in effect between the two processes is apparent.

The fact that both decayed and hydrolysed wood contain a material insoluble in water, but soluble in 1 % sodium hydroxide, which cannot be determined as cellulose, lignin, or pentosans, tends to strengthen the conviction that, in its early stages at least, fungal decay of the brown rot type is in effect an acid hydrolysis. The only possible objection to this conclusion is to be found in the apparently greater solubility of decayed wood in alkali. Hawley and Campbell [1927] emphasise that the alkali solubility of decayed wood is always greater than that of wood that has been hydrolysed to the same extent (as shown by equal loss in weight) but, all things considered, this is only to be expected. Whereas in the hydrolysis of wood with dilute acid all the water-soluble material is removed, wood that has been decayed still contains water-soluble material. Where the losses in weight are the same the alkali solubility of decayed wood will always be greater than that of hydrolysed wood in proportion to the amount of water-soluble material present in the former. If, in Table III, the percentage of hot water-soluble material be added to the loss sustained by decay plus cold water washing, and in Table IV deducted from the loss on alkali treatment, decay can be

strictly compared with hydrolysis. This can be illustrated by the following example.

In Table III the loss in weight sustained by one of the samples after 4 months' decay and cold water washing was 8·0 %. If the figure for the remaining water-soluble material be added to this a total of 10·9 % is obtained. This total is approximately equal to the loss obtained by Hawley and Campbell [1927] by hydrolysing Sitka spruce with 0·25 % hydrochloric acid. Furthermore, the losses due to decay, as expressed by a comparison of Tables I and III, agree very favourably with the losses involved in a hydrolysis with acid of the above concentration. Again, if in Table IV the amount of hot water-soluble material be subtracted from the loss due to alkali extraction, this latter is reduced from 22·4 to 19·5 % of the weight of the original wood. Hawley and Campbell [1927] have shown that Sitka spruce wood which has been hydrolysed with 0·25 % hydrochloric acid is soluble in 1 % sodium hydroxide to the extent of 18·2 %. Thus it can be seen that the agreement between the degree of alkali solubility of partially hydrolysed wood and that of partially decayed wood is in reality quite close, when the necessary allowance is made for the amount of water-soluble material in the latter.

With the advanced stages of decay this investigation is not strictly concerned, but it seems probable that the high degree of alkali solubility of the lignin in badly decayed wood is due, not so much to any direct effect of decay on the lignin complex, as to the large increase of surface area of the lignin caused by marked depletion of the carbohydrate components of the wood.

From the foregoing considerations the similarity of the effect on wood between acid hydrolysis on the one hand, and fungal decay of the brown rot type on the other, is so close as to warrant the conclusion that decay of the brown rot type should, in effect, be regarded as an acid hydrolysis.

Summary.

1. The effect of partial decay on the wood of Sitka spruce caused by *Trametes serialis* Fr. has been examined. This effect is typical of that produced on wood by fungi of the brown rot type.

2. The effect of partial decay on the alkali solubility of the wood of Sitka spruce has been examined, and reasons are given for concluding that, since this effect is of the same order as that produced in the same species of wood by acid hydrolysis, decay of the brown rot type should, in effect, be regarded as an acid hydrolysis.

The authors wish to express their indebtedness to Prof. F. Soddy for facilities afforded in the Old Chemistry Department, Oxford, to Sir James Irvine for helpful criticism and advice, to Messrs K. St G. Cartwright and W. P. Findlay for carrying out the fungal inoculations, and to R. S. Pearson, Esq., for permission to publish these results.

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LXV. THE NATURE OF THE SUGAR RESIDUE IN THE HEXOSEMONOPHOSPHORIC ACID OF MUSCLE.

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(*Received May 10th, 1929.*)

Embden and Zimmermann [1924] have described the isolation from press-juice of rabbit muscle of a hexosediphosphoric acid which was obtained in the form of its neutral brucine salt. They experienced some difficulty in attempting its isolation owing to the presence of free phosphoric acid in their press-juice. Ultimately they eliminated the free phosphoric acid by employing a biological synthetic process involving the use of the fluoride ion which is known to further the synthesis of hexosephosphoric acid from carbohydrate and phosphates. Later Embden and Zimmermann [1927, 1], employing a somewhat different procedure and omitting the fermentative resynthesis, obtained from rabbit muscle pulp a hexosemonophosphoric acid. They were unable to detect the presence of a hexosediphosphoric acid.

In the present investigation it was desired to obtain a supply of the muscle hexosephosphoric acid (frequently referred to as "lactacidogen") in order to study the nature of the sugar residue present in this interesting intermediate substance. The two methods of Embden and Zimmermann were therefore both investigated as a means of obtaining one or other of the two hexosephosphoric acids, our immediate purpose being to obtain that which could be demonstrated to be a normal constituent of the muscle.

The earlier work of Embden and Zimmermann has been fully confirmed and we have obtained from rabbit muscle press-juice the neutral brucine salt of hexosediphosphoric acid identical in all respects with that obtainable from yeast fermentation and now shown by Robison and Morgan [1928] and by Morgan [1929] to be γ-fructose-1 : 6-diphosphoric acid. Similarly, employing the later methods of Embden and Zimmermann, we have likewise obtained only a hexosemonophosphoric acid and have been unable to detect the presence of any of the di-acid ester. It seemed possible that the different results obtained in the two processes of extraction might not be wholly ascribable to the omission of the fermentative resynthesis in the later method, but might in part be due to the other modifications introduced at the same time. It was therefore decided to check this point by employing the first method of extraction, omitting only the addition of sodium fluoride, glycogen and sodium bicarbonate, the reagents used in the fermentative resynthesis. There was

obtained only a hexosemonophosphate identical with the product obtained by the second method of extraction. No trace of diphosphoric acid was detected. The authors are therefore of opinion that the carbohydrate-phosphoric acid ester normally obtainable from resting muscle and regarded as "lactacidogen" consists of hexosemonophosphoric acid. The material used in the present investigation was therefore prepared from the mono-acid ester obtained by Embden and Zimmermann's second method of extraction.

Hexosemonophosphoric acid has been isolated not only from the muscles of rabbits but also from those of the goat and donkey. The yields obtained from the latter sources were lower than those obtained from the rabbit, details being given in the experimental section. This may in part be due to the enzymic breakdown of the compound owing to the necessarily longer time required for the preliminary extraction, but it is our opinion that the poorer yields cannot be wholly ascribed to this cause. The extraction was carried out with all possible speed and the cooling arrangements were as efficient as in the rabbit muscle extractions. It appears possible that the hexosemonophosphoric acid content of the muscles of the larger and more slowly moving animals may normally be less than that of the rapidly contracting muscles of the rabbit. During the course of the rabbit muscle extractions adenylic acid was obtained as has been recorded by Embden and Zimmermann [1927, 2], but this compound was obtained only in very small yield from the muscles of the larger animals. On the other hand, considerable quantities of inositol were obtained from the muscles of the goat and donkey, whilst it was not isolated from any of the rabbit muscle extracts. The best yield of hexosemonophosphoric acid obtained in the present work was 9 g. (37 g. of the neutral brucine salt) from 7 kg. of rabbit muscle, corresponding to 0·13 % of the weight of fresh muscle.

In the experimental section there is described the preparation of free hexosemonophosphoric acid *via* the barium salt, from the recrystallised brucine salt in which form it was first isolated. The acid showed $[\alpha]_{5461} + 33{\cdot}6°$, which is in good agreement with that quoted by Embden and Zimmermann for their preparation, namely $[\alpha]_D + 29{\cdot}5°$. Employing the Willstätter-Schudel hypoiodite method of oxidation these workers found that the sugar constituent of their acid consisted of 91 % of aldose in one preparation and 93 % of aldose in another. Our preparations showed 90 % of aldose sugar. From the free acid a crystalline osazone was obtained without loss of the phosphoric acid grouping. It melted in the vicinity of 145–147° with decomposition.

After a series of preliminary investigations which need not be detailed here it was decided that the nature of the sugar residue present in muscle hexosemonophosphoric acid could best be decided by oxidation to the corresponding hexonic acid and subsequent removal of the phosphoric acid group. The first step was effected with the use of bromine and the second with 10 % sulphuric acid in a sealed tube at 100°. There was finally obtained the calcium salt of a hexonic acid which was free from phosphorus and from reducing material. The free hexonic acid prepared from the calcium salt had $[\alpha]_{5461} + 10{\cdot}9°$, and

after heating for 1 hour at 65°–70° it showed $[\alpha]_{5461} + 24{\cdot}0°$. This change is characteristic of a hexonic acid undergoing lactonisation and these figures are in good agreement with those obtained for pure preparations of gluconic acid subjected to the same treatment. We are therefore of the opinion that the aldose of hexosemonophosphoric acid is *d*-glucose.

Experimental.

Isolation of hexosediphosphoric acid from rabbit muscle press-juice.

The first pair of rabbits used were not specially fed, but in the later experiments the rabbits were fed with generous supplies of oats for at least 3 days immediately before they were killed. Some were killed by a sharp blow on the back of the neck followed by immediate decapitation. A disadvantage of this method of killing is the resulting violent twitching of the muscles, and in the later experiments the animals were asphyxiated with coal gas until the corneal reflex failed and then decapitated. The pelt was removed as quickly as possible and the musculature was excised and minced in an ice-cold machine. The subsequent steps in the extraction were essentially those of Embden and Zimmermann [1924]. A Buchner oil press was used, and the juice was collected under pressures up to 150 kg./cm.^{2} The cloth used was made of camel hair and was supplied by Messrs Premier Filterpress Co., Ltd., London. The yields obtained are indicated by the following typical results.

I. 2 rabbits, rather small and not specially fed. Juice obtained, 415 cc.; recrystallised brucine salt, 1·5 g.

II. 2 rabbits, fed on previous day with oats. Juice obtained, 500 cc.; recrystallised brucine salt, 2·5 g.

III. 2 rabbits, fed for 3 days with oats. Juice obtained, 540 cc.; recrystallised brucine salt, 3·5 g.

The brucine salt was twice recrystallised from aqueous methyl alcohol and dried *in vacuo* over sulphuric acid and finally at 56° over phosphorus pentoxide in a high vacuum. It gave satisfactory analyses and showed $[\alpha]^{17°}_{5461} - 30{\cdot}7°$ ($c = 1{\cdot}01$ %). For comparison purposes Mr W. J. T. Morgan, of the Lister Institute, London, kindly prepared a specimen of brucine hexosediphosphate from yeast fermentation and this showed in methyl alcohol $[\alpha]^{18°}_{5461} - 30{\cdot}7°$, thus confirming the identity of the two products. When heated rapidly our twice recrystallised preparation melted with considerable charring between 170° and 180°.

Isolation of hexosemonophosphoric acid from the press-juice of rabbit muscle.

For reasons already given in the introduction it was decided to isolate from rabbit muscle and identify the hexosephosphoric acid or acids, using the same method as that used for the isolation of hexosediphosphoric acid, omitting only

the fermentative resynthesis involving the addition of glycogen and sodium fluoride in the presence of sodium bicarbonate.

The muscle juice from six normal rabbits was collected in as short a time as possible and in efficiently cooled vessels. With one exception, when the rabbit was killed by a sharp blow, all the animals were asphyxiated with coal gas. They had all been previously fed on oats for at least 3 days. The total volume of juice obtained was 1350 cc. At the stage when deposition of brucine hexosediphosphoric acid should have occurred no deposition of crystals was noted even after prolonged scratching and on allowing the solution to stand in the ice-chest overnight. The following day slow crystallisation began and after 2 days some clusters of small crystals separated from the solution. These were obtained in very small amount and were found to contain a pentose. They were subsequently proved to consist of the brucine salt of adenylic acid, to which reference will be made in a later communication. The solution was treated with several volumes of acetone in order to complete the deposition of the brucine salt of adenylic acid. The solid obtained was extracted with a small volume of anhydrous methyl alcohol and filtered. The filtrate was concentrated to dryness and again extracted with methyl alcohol and filtered. The solution was again taken to dryness and the crystalline residue was dried to constant weight. It had P = 2·83 % (calc. for brucine hexosemonophosphate 2·96 % and for the hexosediphosphate 3·21 %), and showed $[\alpha]_{5461}$ − 19·4° in methyl alcohol. Our later preparations of brucine hexosemonophosphate showed in the same solvent $[\alpha]_{5461}$ − 20·3°. In this experiment therefore there was obtained no evidence of the presence of a hexosediphosphoric acid and it is concluded that the latter is either entirely absent or present only in very small amounts in normal muscle press-juice.

Isolation of hexosemonophosphoric acid from rabbit, goat and donkey muscle.

The method of extraction was that already referred to as Embden and Zimmermann's second method, with minor modifications which need not be detailed.

Embden and Zimmermann [1927, 1] report an average yield of 11 g. of unrecrystallised brucine hexosemonophosphate from twelve rabbits yielding 6 kg. of muscle. The best yield obtained in the present work was 23 g. of brucine hexosemonophosphate from twelve rabbits yielding 7 kg. of muscle, whilst in this case the basic lead acetate fraction gave a further yield of 14 g. of the brucine salt. That our yields are materially higher than those recorded by Embden and Zimmermann is doubtless in part due to the fact that we subjected all protein residues to high pressure extraction, using the Buchner press.

After one recrystallisation brucine hexosemonophosphate gave in water $[\alpha]_{5461}$ − 29·9° and in anhydrous methyl alcohol − 20·3°. The recrystallised salt after drying *in vacuo* over phosphorus pentoxide showed signs of softening at 145°, melted at 155° and decomposed at 158–160°.

In order to obtain larger supplies of hexosemonophosphoric acid extracts were made from the minced muscles of larger animals, namely two goats and one donkey. The yields were disappointingly small considering the size of the animals. The animals were fed for some days on oats and hay and were killed with a "humane killer" and in no case was any muscle twitching observed after the death of the animal. The extractions were carried out in a manner identical with that used in the case of the rabbits and the operations were expeditiously carried through and the bulky solutions were efficiently cooled with ice. The protein precipitates were filtered on large stoneware filters, using muslin to give added support to the filter-papers. The work was continued without a break until the deproteinised filtrates had been freed from mercury and excess hydrogen sulphide and then almost neutralised with 33 % sodium hydroxide. The results may be summarised as follows.

Goat I ♀. All muscles minced and in acid bath in 25 minutes. Temp. rose for few minutes to 10°, but was quickly reduced to 2°. Weight of muscle, 6 kg.

Via normal lead acetate precipitate, 3·3 g. of brucine salt and 1·3 g. inositol.

Via basic lead acetate precipitate, 8·8 g. brucine salt and 1·8 g. inositol.

Goat II ♀. All muscles minced and in bath in 20 minutes. Temp. rose for few minutes to 10°, but solution was quickly cooled to 2°.

Weight of muscle, 10·5 kg.

Via normal lead acetate precipitate, 1·4 g. brucine salt and 0·7 g. inositol.

Via basic lead acetate precipitate, 3·0 g. brucine salt and 1·3 g. inositol.

Donkey ♂. All muscles minced and in acid bath in 50 minutes. Cooling arrangements efficient. Temp. quickly reduced to about 3° or 4°. Weight of muscle, 30·2 kg.

Via normal lead acetate precipitate, 3·4 g. brucine salt and 0·2 g. inositol.

Via basic lead acetate precipitate, 2·1 g. brucine salt and 4·5 g. inositol.

The brucine salts after recrystallisation gave the same optical rotation and melting point as those obtained from rabbit's muscle. Further, the free hexosemonophosphoric acid prepared *via* the barium salt from the mixed brucine salts obtained from rabbit, goat and donkey gave the same optical rotation.

A point which has been referred to in the introduction is the absence of adenylic acid and the presence of inositol in these later extractions. Using Bial's reagent it was established that the pentose-containing constituent was present only in very small amounts and it was found impossible to isolate any adenylic acid either from the goats or from the donkey. On the other hand, there was obtained, from the aqueous-acetone solutions from which adenylic acid was deposited in the case of the rabbit muscle extractions, a white crystalline compound which was recrystallised from hot water, in which it was readily soluble. It gave a negative result on testing with Molisch's reagent, did not reduce Fehling's solution, contained no nitrogen or pentose constituent and possessed a definitely sweet taste. It was found to be optically inactive, which indicated with the previous tests that this crystalline compound was *i*-inositol. This was confirmed by a melting point, the dried substance melting at 224–225°

with preliminary softening, and also by a positive Sherer's test. After two recrystallisations from water the substance still retained a slight trace of organically combined phosphorus (indicated with Bell-Doisy reagents).

Preparation of barium hexosemonophosphate.

Barium hexosemonophosphate was prepared from the recrystallised brucine salt by the method adopted by Embden and Zimmermann. The barium salt was precipitated as an amorphous solid by adding a saturated solution of barium acetate in 80 % methyl alcohol to a 10 % aqueous solution of the brucine salt. After standing for a few hours in the cold the salt was collected on a small Büchner funnel, washed with 80 % methyl alcohol, and finally with pure methyl alcohol. The product was then thoroughly dried over phosphorus pentoxide in a vacuum. It was ground in a mortar with a little water when most of the barium salt dissolved, leaving a less soluble yellowish residue. The barium salt was then reprecipitated with methyl alcohol and treated as before. This treatment was repeated at least once, sometimes twice, with all samples of the barium salt prepared. In this way the final product was freed from brucine and also from the more insoluble constituent with which it is initially associated. In one preparation it was found advantageous to warm for some time with a little norite. Again, in another preparation a considerable quantity of the less soluble material was separated from the barium salt by extracting with warm water. But it is essential that the precipitated product be thoroughly dried before a further aqueous extract is made, otherwise it is impossible to obtain the barium hexosemonophosphate in a state of purity.

Embden and Zimmermann record having obtained evidence of iron and phosphorus in one of the less soluble yellowish residues already referred to. The authors obtained negative results in testing for iron, except in one sample when a very faint blue colour was developed with potassium ferrocyanide, after thoroughly digesting the material with hot concentrated sulphuric acid and 30 % hydrogen peroxide. The brucine used in this instance, however, was itself found to contain traces of iron and it is therefore not thought that the iron found in the residue from the barium hexosemonophosphate has necessarily any biological significance. Repeated extraction of the less soluble material with water left the residues progressively poorer in phosphorus. Further, barium salts (especially those prepared from brucine salts obtained *via* the basic lead acetate precipitates) contaminated with this extraneous material gave low analytical figures and the free hexosemonophosphoric acid subsequently obtained showed a lower specific rotation and gave lower reduction values. These syrupy residues, which on drying form a glass, are therefore not of the nature of sugar phosphates and were not further investigated.

Hexosemonophosphoric acid.

The optical rotation of the free hexosemonophosphoric acid was determined in the following manner. A weighed amount of the dried barium salt was dissolved in water and the barium precipitated by the addition of a slight excess of sulphuric acid. The barium sulphate was centrifuged off, and the clear supernatant solution decanted into a suitable measuring flask. The barium sulphate precipitate was then washed twice with small quantities of water and after centrifuging these were added to the solution, which was finally made up to a definite volume. The free acid, liberated in the above manner from carefully dried barium hexosemonophosphate (Ba = 34·5 %, P = 7·8 %, calc. for $C_6H_{11}O_5(PO_4Ba)$, Ba = 34·7 %, P = 7·85 %), gave $[\alpha]^{17°}_{5461}$ + 33·0° in water (c = 0·770).

The hexosemonophosphoric acid obtained in the same manner from the mixed extracts of rabbit, goat, and donkey muscle, gave $[\alpha]_{5461}$ + 33·6° in water (c = 2·93). As indicated above, specimens of barium hexosemonophosphate which gave low analytical figures, gave rise to a free acid with lower specific rotation and, further, correspondingly low copper reduction values. Thus one preparation of barium hexosemonophosphate gave a specific rotation for the free acid of $[\alpha]_{5461}$ + 23·1 (calc. from the weight of barium salt originally taken). On regrinding some of the barium salt with water a small amount of insoluble material remained. After reprecipitation with methyl alcohol the salt was carefully dried as before. The barium salt obtained still had a low phosphorus content (P = 7·25 % calc. 7·85), but the rotation was higher than the previous value, being $[\alpha]_{5461}$ + 26·5°.

The copper-reduction value of hexosemonophosphoric acid was then determined, employing the method described by Bertrand. The values obtained were compared with those of glucose.

$$\text{Ratio } \frac{\text{Hexosemonophosphoric acid (calc. as hexose)}}{\text{Glucose}} = 0{\cdot}74.$$

Formation of barium methylhexosidemonophosphate.

About 2·1 g. of barium hexosemonophosphate was dissolved as quickly as possible in 55 cc. of anhydrous methyl alcohol containing 1 % hydrochloric acid. Polarimetric observations were made on the solution and the observed rotations in a 1 dm. tube are recorded below. After the first 8 hours the solution was maintained at 25° in a well-stoppered flask. A short time after the first observation was made barium chloride began to separate out and it was then necessary to filter the solution immediately before observing the optical rotation. The solution still reduced Fehling's solution 74 hours after it was prepared. Tested after 97 hours the solution showed no reduction with Fehling's solution. Observed rotations $[\alpha]_{5461}$: + 0·41° (8 hrs.), 0·29° (12 hrs.), 0·38° (32½ hrs.), 0·51° (73½ hrs.), 0·57° (98½ hrs.), 0·59° (121½ hrs.), 0·61° (146 hrs.), 0·64° (168½ hrs.). It will be noticed that initially there is a fall in

the observed rotation followed by an increase, which gradually approaches a maximum. These changes are evidently due to the different rates of formation of the α- and β-isomers of the methylhexosidemonophosphate.

An attempt was made to isolate the barium methylhexosidemonophosphate and then to dephosphate this compound with a preparation of bone enzyme, using the method described by Morgan [1927] and applied by him to the investigation of hexosediphosphoric acid from yeast. But the different solubilities of the corresponding salts of the hexosemonophosphoric acid make this method unsuitable, especially with small amounts of material.

An osazone was prepared from the hexosemonophosphoric acid without liberation of free phosphoric acid. 0·3 g. of the barium salt was treated with a slight excess of sulphuric acid and after removing the precipitated barium sulphate the solution was heated for half an hour in a boiling water-bath with 0·5 g. of phenylhydrazine hydrochloride and 1 g. of sodium acetate. Crystallisation commenced immediately the solution was cooled. Viewed under the microscope the crystals presented the appearance of clusters of flat needles. They began to soften at 140° and melted at 145°–147° with decomposition.

Willstätter-Schudel oxidation.

Employing the technique described by Goebel [1927] for the Willstätter-Schudel oxidation with hypoiodite, hexosemonophosphoric acid gave values indicating that 90 % of the hexose in this acid is of an aldose nature.

After oxidation by this method, the solution still reduced Fehling's solution. This is interpreted as indicating the presence of some 10 % of a ketosemonophosphate admixed with 90 % of aldosemonophosphate.

Preparation of hexonic acid from hexosemonophosphoric acid.

About 0·8 g. of hexosemonophosphoric acid dissolved in 23 cc. of water was treated with 0·75 g. of bromine. After standing for 16 hours at air temperature a portion of the solution, after aerating to free from bromine, still reduced Fehling's solution. The solution was still slightly reducing to Fehling's solution after 9 hours in a water-bath kept at 30°. A micro-determination of sugar was then made employing the method of Shaffer and Hartman. Later determinations, after the solution had been maintained at 30° for a further period of 11 hours, gave a reduction value only slightly less than that obtained previously. From these determinations about 0·04 g. (calc. as glucose) remained unoxidised. It may be noted here that only slight traces of free phosphoric acid could be detected in the solution after the above oxidation. The solution was aerated to free from bromine and then sufficient hydrobromic acid added to give a 14 % solution of the acid. This solution was heated in a sealed tube at 85° for 20 hours. On opening the tube, free phosphoric acid was found to be present in small amount only. Sulphuric acid was then added sufficient to give a 10 % solution of this acid, and then after resealing the solution was

heated for 7 hours at 100°. The resulting perfectly clear solution contained considerable amounts of free phosphoric acid. It was neutralised with silver oxide, and the filtrate subsequently obtained was freed from silver with hydrogen sulphide. After aeration the solution was found to be free from phosphorus, non-reducing, and optically active in the dextro-sense. The solution was heated to 75°, calcium carbonate added, and the solution well stirred for several minutes. After cooling, the solution was filtered and concentrated to dryness at 45°. The solid residue was then extracted with a small volume of hot water and filtered. The filtrate still contained calcium sulphate which was precipitated by the addition of an equal volume of absolute alcohol. After standing for some days the solution was filtered and concentrated under reduced pressure. Calcium sulphate was still present in minute quantities, and the solution was again precipitated by adding several volumes of alcohol. The precipitate was collected and extracted with about 10 cc. of water. The small amount of insoluble material was centrifuged off and the clear supernatant solution again precipitated with several volumes of alcohol. The perfectly white product was collected and dried overnight in an evacuated desiccator containing phosphorus pentoxide. The dried material gave a calcium content of 9·2 % (micro-determination) (calc. for $(C_6H_{11}O_7)_2Ca$, 9·3 %).

0·118 g. of the calcium salt was dissolved in water and one drop of concentrated hydrochloric acid added to liberate the free hexonic acid. The volume was made up to 10 cc. thus giving a 1·06 % solution of the free hexonic acid.

This solution gave $[\alpha]^{18°}_{5461} + 10·9°$.

After the solution had been maintained at 50° for 1½ hours the rotation increased, and on cooling gave $[\alpha]^{21°}_{5461} + 14·6°$.

On the addition of a small drop of concentrated hydrochloric acid the rotation dropped immediately to its previous value. The solution was reheated for an hour at 65°–70° and then gave $[\alpha]^{68°}_{5461} + 26·8°$.

The temperature of the jacketed tube was then speedily reduced to 24°. The solution then showed $[\alpha]^{24°}_{5461} + 24·0°$.

A reading taken 15 minutes later showed identically the same value. These results are in good agreement with those recorded by Tollens [1914] for gluconic acid. The calcium salt has $[\alpha]_D + 10°$ and prepared directly from the calcium salt the equilibrium mixture of free acid and lactone has $[\alpha]_D + 23·4°$ after the solution has been heated for some time.

Summary.

(1) The hexosephosphoric acid of normal muscle press-juice from rabbit, goat and donkey is a monophosphoric acid. The diphosphoric acid, identical with that of yeast fermentation, is only obtained when the fermentative resynthesis using sodium fluoride is employed.

(2) The carbohydrate residue of muscle hexosemonophosphoric acid consists of 90 % of aldose, identified as *d*-glucose, and 10 % of ketose.

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LXVI. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

IV. A COMPARISON BETWEEN GLUCOSE AND GLYCOGEN IN RESPECT OF LACTIC ACID FORMATION AND PHOSPHORIC ESTER ACCUMULATION.

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In this paper are described eight experiments with individual extracts prepared in the same manner from eight healthy cats. In each case the reaction at the start of the incubation was between p_H 6·9 and 7·1; glucose and glycogen were used respectively in a concentration of 0·5 %. These eight experiments thus constitute a good series for the purpose of detecting any difference between glucose and glycogen with regard to the apparently related reactions of lactic acid formation and phosphoric ester accumulation.

Experimental.

The extracts were prepared from perfused muscle by the method described in a previous paper [Stiven, 1928]. The incubation was conducted in an atmosphere of nitrogen as described therein. The purity of the glycogen was estimated by hydrolysis to glucose and in weighing out the glycogen for making up the stock 10 % solution to be added to the extract, a correction was made for the purity as indicated by the yield of glucose. The lactic acid was estimated by the aeration method of Friedemann, Cotonio and Shaffer [1927]; the "inorganic" phosphorus by the colorimetric method of Fiske and Subbarow [1925]. Both lactic acid and "inorganic" phosphorus were estimated at intervals of 10 minutes up to the end of the first hour and thereafter at 90 minutes and 120 minutes from the start of the incubation. In this way the course of the reactions could be followed at short intervals and any resemblance or difference between the behaviour of these two carbohydrates could be detected.

The term "inorganic" phosphorus means in this paper the sum of the phosphagen phosphorus and the true inorganic phosphorus. In every experiment the lactic acid and the "inorganic" phosphorus changes were investigated in an aliquot sample of the extract to which no carbohydrate had been

added. In this "plain" extract the "inorganic" phosphorus increases rapidly during the first 40 minutes of the incubation but thereafter very slowly. In the case of the extracts to which carbohydrate was added this increase of "inorganic" phosphorus does not take place until the incubation has proceeded for 40 to 60 minutes if lactic acid is being formed from the added carbohydrate. In the early period of the incubation a decrease of the "inorganic" phosphorus may in some cases be observed. This decrease of "inorganic" phosphorus indicates that the formation of phosphoric ester is proceeding at a greater rate than the breakdown of the ester. The amount of this decrease between any two intervals is thus a measure of the excess of ester formed over that broken down in that interval and is called here ester accumulation.

Table I. *Lactic acid formation and phosphoric ester accumulation from glycogen and glucose both present to* 0·5 %.

Experiment	Lactic acid formed (mg. per 100 cc. extract) in 20 mins.	40 mins.	60 mins.	120 mins.	Maximum decrease of "inorganic" phosphorus (mg. per 100 cc. extract)	Time of maximum ester accumulation (mins.)
252 Glycogen	106	170	227	264	21	10
Glucose	118	213	300	427	3	10
254 Glycogen	72	98	114	130	25	20
Glucose	78	137	162	186	2	20
255 Glycogen	80	143	171	207	35	40
Glucose	84	162	224	295	0	—
256 Glycogen	107	157	195	284	29	10
Glucose	70	141	202	321	2	60
257 Glycogen	102	154	191	233	13	10
Glucose	64	104	113	119	0	—
260 Glycogen	127	183	229	260	38	20
Glucose	145	229	247	250	1·5	10
261 Glycogen	101	154	192	301	46	40
Glucose	79	125	192	332	1·5	50
262 Glycogen	90	167	216	252	6	10
Glucose	69	150	222	350	0	—

From the results in Table I it will be noticed that decidedly greater ester accumulation took place in every case with glycogen. With glucose it was very feeble and in some cases never took place at all. The striking difference between glucose and glycogen in this respect is not due to a less extensive formation of lactic acid from the hexose nor to a lower rate of formation of the acid. These results seem to indicate that at the start of the incubation, when the concentration of the glycogen is high, the formation of phosphoric ester generally proceeds at a greater rate than the subsequent removal of the phosphoric acid groups. With glucose, on the other hand, it would appear that these two opposing and concomitant reactions almost balance, and from an examination of a great many extracts it has been noticed that the greater the rate of glycolysis the more does the rate of ester formation tend to exceed that of the breakdown. If lactic acid originates only from carbohydrate that

has been through the intermediate stage of phosphoric ester, then it is difficult to understand why in the case of glycogen the ester should sometimes accumulate to the extent it does, particularly in those cases where the rate of lactic acid formation is greater than with glucose, indicating to all appearances a more rapid hydrolysis of the ester. It is conceivable, however, that the ester which accumulates in the glycogen samples does not have the same structure and hence does not yield the same end product as the intermediate ester formed from glucose. This point is under investigation and will be discussed in subsequent papers.

Another point deserves consideration. It will be noticed that the lactic acid formation from glucose varies greatly from one experiment to another. In some cases it is greater with glucose than with glycogen and in other cases it is not. It might be argued that in a series of experiments like the above where the conditions were kept as uniform as possible, this variation might be due rather to faulty technique than to a real difference produced by some unknown factor. The following experiment shown in Table II tests this point. 2 kg. of cat muscle were ground up and extracted in the usual manner, and the final volume of extract, 900 cc., was divided up into three portions of 180 cc. To each of these were added 9·5 cc. of 10 % glucose solution. The "inorganic" phosphorus and the lactic acid were estimated at the intervals indicated.

Table II, Exp. No. 265. *"Inorganic" phosphorus concentrations and lactic acid increases in three aliquot portions of the same extract, all containing* 0·5 % *glucose.*

Time in mins.	"Inorganic" phosphorus concentration (mg. per 100 cc.)			Increase in lactic acid (mg. per 100 cc.)		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
Start	71·3	71·0	71·0	—	—	—
10	70·5	70·5	70·5	36	36	38
20	71·0	71·0	71·0	72	75	72
40	72·3	72·3	72·1	166	167	166
60	75·7	76·0	76·0	219	222	228
90	82·3	82·9	82·9	291	308	311
120	89·5	89·5	89·5	313	325	327

The above results indicate the extent of the variation that is to be expected. The lactic acid values show greater fluctuations than the phosphorus values but these are not of sufficient magnitude to account for the erratic nature of the results in Table I. These must be due to some unknown factor connected with the preparation of the extract or even with the animals. In this connection it has been found that tame rabbits, as compared with wild rabbits or cats, give invariably most disappointing results as regards lactic acid formation from glucose.

SUMMARY.

Extracts from the muscles of eight cats have been examined for lactic acid formation and phosphoric ester accumulation from both glucose and glycogen. In the case of glucose the extent of the ester accumulation is very small compared with that from glycogen even when the rate of lactic acid formation from glucose is greater than from glycogen. Both the rate and extent of the lactic acid formation from glucose are, generally speaking, greater than from glycogen, but in this respect there is a great variation from one extract to another. It is shown that this variation is not due to faulty technique in the manner of incubation or in the analytical procedure, but is due to some factor as yet uncontrolled.

In concluding, the author wishes to express his thanks to Prof. E. Waymouth Reid, F.R.S., for his assistance in the perfusing of the cats.

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